Development and Application of *In Vitro* Compartmentalized Devices to Study Axonal Injury

by

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Abstract

Regeneration of injured peripheral nervous system (PNS) axons is a widely observed, but incompletely understood phenomenon. While PNS axons are capable of spontaneous regeneration, functional recovery may be difficult to achieve. The complex *in vivo* environment provides a challenging setting to examine axonal injury and regeneration, making reductionist *in vitro* approaches necessary. Cell culture-based injury platforms allow for injury of individual axons while organotypic platforms more closely resemble the *in vivo* injury environment. This dissertation presents the development and application of novel compartmentalized *in vitro* platforms, first presenting cell culture devices and culminating with an organotypic (slice culture) platform, to enable study of the site of action of degenerating and regenerating compounds on PNS axons. First, microfluidic devices were utilized to determine that the chemotherapeutic agent paclitaxel is most destructive when applied to the axon, and that this chemotherapy-induced axonal degeneration can be limited by application of erythropoietin to either the cell body or axon, indicating that local mechanisms can be counteracted through cellular mechanisms. Next, regenerative effects of

ABSTRACT

members of the glial cell-line derived nerve growth factor (GDNF) family of ligands (GFLs) were investigated in a microfluidic physical injury model. All tested GFLs enhanced regeneration regardless of site of application, with GDNF showing the most enhancement. Concurrent application of a retrograde transport blocker with GDNF diminishes this regenerative effect, indicating the importance of cellular rather than local mechanisms. Finally, a two-compartment culture device that enables control of the local environment of regenerating adult motor axons was developed and optimized, and functional compartmentalization demonstrated. This device is the first of its kind and allows for tailoring of the immediate environment of adult motor axons regenerating within the three-dimensional structure of peripheral nerve. The ability to study axonal injury and regeneration in such highly tailorable environments will lead to more comprehensive understanding of injury and regeneration, aiding eventual goals of improving reinnervation accuracy and surgical outcomes.

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I would not be who I am without my experiences here and everyone whom I have thanked. I am looking forward to my next journey. Thank you all so much!

Dedication

This thesis is dedicated to my parents, family, and friends for their unconditional love, support, and encouragement.

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Chapter 1

Introduction

1.1 Overview

Peripheral axon injury and regeneration is widely observed, yet poorly understood. The inherent complex design of the nervous system necessitates the use of reductionist *in vitro* devices that enable a systematic probing of cellular processes. Here I will provide context for my thesis by providing a general overview of nervous system structure and anatomy, an overview of compartmentalized devices, and a discussion of current approaches and challenges to treating peripheral nerve injury. I will conclude this overview chapter with a discussion of my goals, specific aims, and the organization of my dissertation. Separate abstracts and introductions precede Chapters 2-6 in order to provide a more specific overview relevant to those chapters.

1.2 Nervous System Structure

An understanding of the general organization and components of the nervous system is necessary for insight into difficulties that may arise during nerve injury and regeneration. The nervous system is categorized into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS serves as a control center, conducting and interpreting signals, and consists of the brain and spinal cord, while the PNS consists of nerves, both motor (efferent) and sensory (afferent), that transmit signals between the CNS and the rest of the body. Neurons are the functional units of the nervous system and do not undergo mitosis, while glial cells are the support cells of the nervous system and much more plentiful than neurons. Schwann cells are the principle glial cells of the PNS, while oligodendrocytes, astrocytes, and microglia are the glial cells of the CNS. These cells will be gone into further detail in the next chapter.

The spinal nerves of the PNS originate from the spinal cord, a schematic of which can be seen in Figure 1.1. The spinal cord contains central butterfly-shaped grey matter made up of the cell bodies of excitatory neurons, CNS glial cells, and blood vessels. Surrounding the grey matter is insulting and protective white matter consisting of axons and glial cells of the CNS. Oligodendrocytes myelinate the axons, while astrocytes help form the blood-brain barrier. Axons project from the white matter in fascicles, travel through the PNS-CNS transition zone, and enter the PNS. In the transition zone, the glial cells of the CNS are separated from the glial cells of the PNS.

The nerves exiting the sides of the spinal cord can be divided into subgroups, with the ventral root carrying motor signals from the CNS and the dorsal root carrying sensory information to the CNS. The cell bodies of the sensory neurons are located in dorsal root ganglia (DRG) located along the dorsal root while the cell bodies of motor neurons are located within the spinal cord as can be seen in the schematic in Figure 1.1. As axons can be on the scale of meters long in the PNS, the cell bodies of these peripheral neurons may experience drastically different environments from their distal axons. The spinal nerve contains contributions from both motor and sensory neurons and is thus a mixed nerve. Along with the neurons, these peripheral nerves are bundled together with support tissue. Endoneurium is composed mainly of oriented collagen fibers surrounds individual axons, while perineurium is composed primarily of fibroblasts and collagen and surrounds groups of axons to form fascicles. Epineurium is made up loose fibrocollagenous tissue encases fascicles to form a nerve trunk. Capillaries within the support tissue and other penetrating vessels ensure that the nerve trunk is well vascularized.

The neurons that make up a nerve can be further subdivided into the soma (cell body), dendrites, and the axon, as can be seen in Figure 1.2. Signals typically propagate from the axon of one neuron to the dendrite of another, connecting at a synapse . While a typical neuron may have many dendrites, a neuron generally has one axon from one point of origin, although it may later branch. The cell body of a neuron contains its nucleus and is the location of protein synthesis. Dendrites are branched

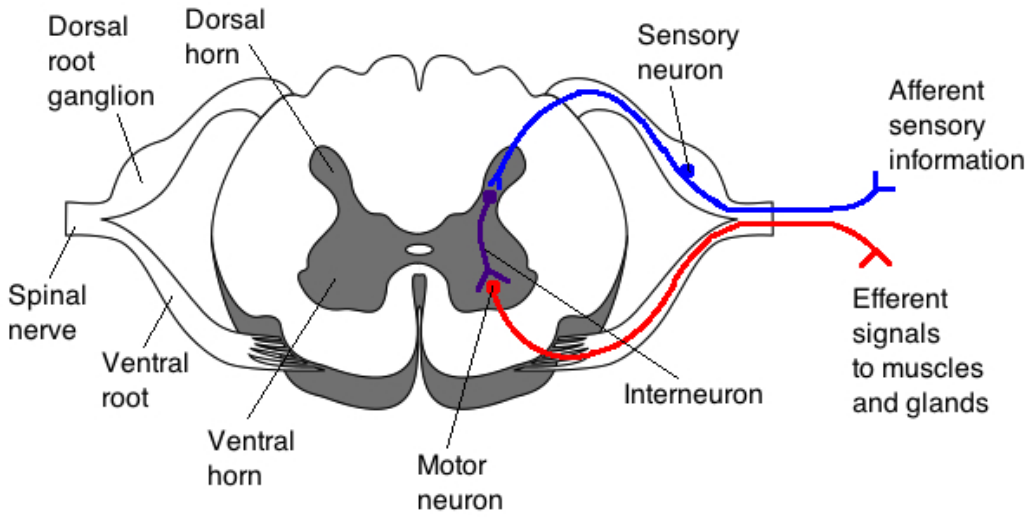

Figure 1.1: Schematic of a transverse section of spinal cord

cellular extensions from the cell body. In contrast to dendrites, axons can be extremely long in the peripheral nervous system and can have lengths on the order of meters. These axons contain cytoplasm and are provided structure by microfilaments and microtubules. Here, the microtubules are important not only with regard to structure, but also to growth and axonal transport. Axonal transport is an essential function, as it involves not only the transport of proteins and organelles from the cell body to the axon, but also for removing waste molecules at the distal axon by the cell body. Axonal transport is accomplished through motor proteins which bind to both the microtubules and potential cargo. Kinesin is responsible for anterograde transport from the soma to the distal axon, while dynein is responsible for retrograde transport from the distal axon back to the soma. Disruption of this axonal transport

can potentially trigger nerve degeneration. Nerve injury neurobiology, and particularly how it differs in the CNS as compared to the PNS, will be discussed in further detail in the next chapter.

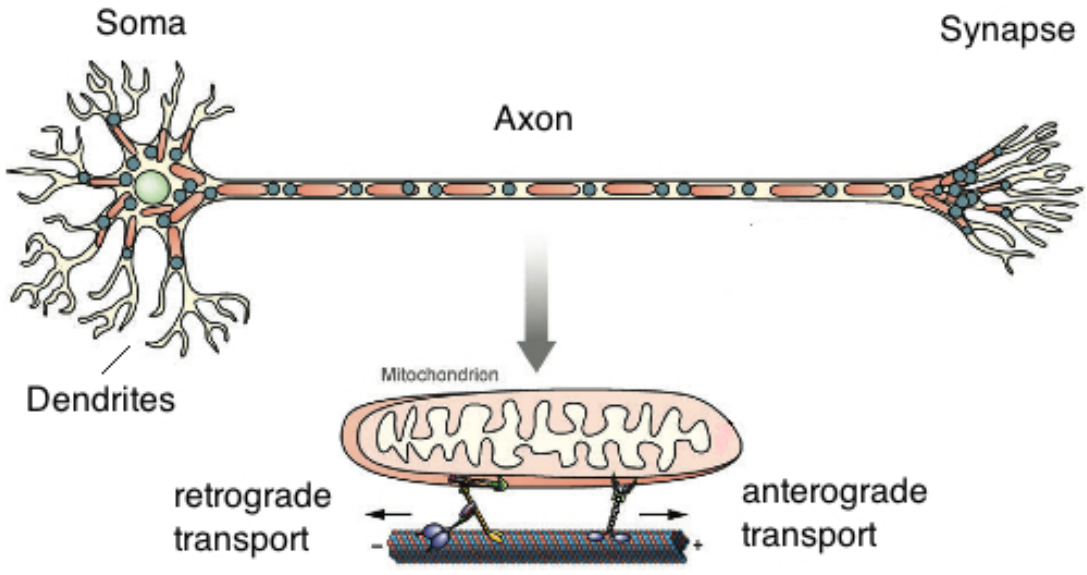

Figure 1.2: Schematic of neuronal structure indicating the important role of microtubules in axonal transport. Modified from [3].

1.3 Compartmentalized Platforms for Neuronal Studies

The Campenot Chamber was the first device to demonstrate individual manipulation of cell bodies and axons in PNS neurons [4]. Demonstrated in the schematic in Figure 1.3, a Teflon divider attached to a collagen coated glass petri dish sealed with

silicon grease served to separate cell bodies from axons, while cuts made in the glass dish served to guide axons. The isolated compartments can then be independently manipulated by adding growth factors or other proteins. These devices allowed for the first site of action studies, but they are prone to leakiness and irreproducibility from device to device. More recent neuronal studies have focused on compartmentalized microfluidic systems fabricated through poly(dimethylsiloxane) (PDMS) soft lithography, which allow for fluidic isolation of axonal chamber from somal chamber through microliter level volume differences between the two chambers, while microchannels between the chambers provide high fluidic resistance that leads to a small but sustained flow that counteracts diffusion [5–8]. These devices are highly reproducible and many can be fabricated easily. The utilization of such a system, with an incorporated physical injury modality, will enable the study of the regeneration environment of single axons. These types of culture systems have high reproducibility and ease of fabrication, and allow for isolation of molecular mechanisms.

Applying the concept of creating dual compartments to organotypic cultures extends the efficacy further. Organotypic cultures are useful for motor neuron studies due to difficulties maintaining these cells in monolayer cultures (which involve plating of dissociated cells) for longer periods [9]. In Figure 1.4 we see a comparison of microfluidic cultures and organotypic cultures to other commonly used commonly used techniques for studying nerve injury. While cell culture devices such as Campenot chambers and their microfluidic counterparts allow for compartmentalized exposure

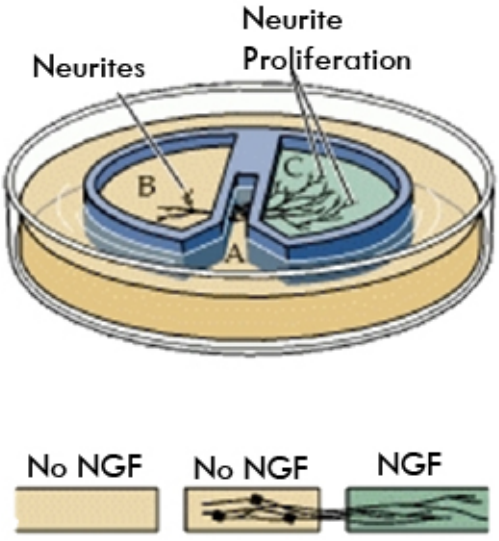

Figure 1.3: The Campenot Chamber, the first compartmentalized device for neuronal cell culture, consisted of a teflon divider attached to a glass petri dish with silicon grease. Neurons can be seeded in one compartment (A), and neurites extend into other compartments by following scratches made on the surface of the glass. These compartments can be individually manipulated (in this example with nerve growth factor (NGF) added to compartment C with B serving as a control). Modified with permission from [1].

through fluidic isolation, these do not reproduce the three dimensional structure of nerve and thus cannot accurately model nerve repair. One of the aims of this thesis is to address the unmet need of a compartmentalized organotypic injury platform.

The Brushart lab has developed an *in vitro* model of adult mammalian nerve repair in an organotypic co-culture system [10]. Organotypic cultures are prepared from nervous tissue without dissociation and thus preserve three-dimensional cytoarchitecture within tissue slices [11]. This concept is applied to spinal cord slices and peripheral nerve, in order to create a co-culture system amenable to nerve repairs. In

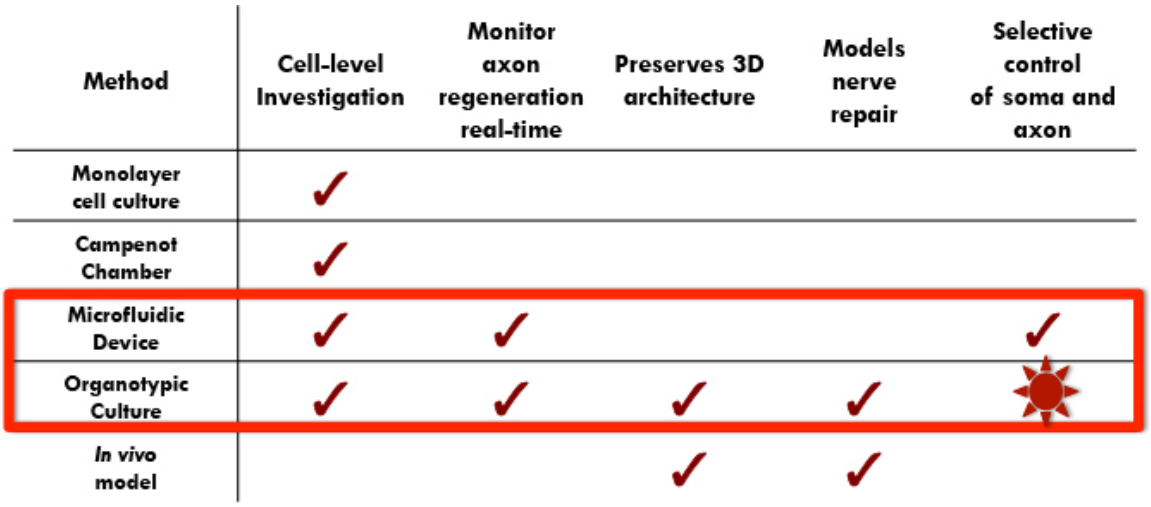

Figure 1.4: Comparison of nerve injury investigation methods. Comparing across multiple commonly used techniques for studying nerve injury from least to most complex (top to bottom), microfluidic cultures and organotypic cultures provide an intermediate complexity between simple monolayer cultures and *in vivo* models while allowing for more precise control and monitoring. The unmet need of an organotypic platform that allows for selective control of the axon versus the soma is addressed in this thesis.

order to isolate the effects of growth factors, there is a need to further modify this system in order to minimize diffusion and allow isolated exposure of growth factors to regenerating axons. While monolayer cell culture devices allow for compartmentalization through fluidic isolation, there are no such devices for the organotypic spinal cord and peripheral nerve co-culture. The development of such a device would allow for the study of axon specific mechanisms of motor axon regeneration by allowing for control of the local environment of regenerating axons within an *in vitro* device that more closely replicates an *in vivo* three-dimensional environment.

1.4 Current Approaches and Challenges

The current treatment for peripheral nerve injury is end-to-end surgical reconnection for smaller defects, or reconnection with an autologous nerve graft for larger ones. Direct reconnection cannot be used for larger defects due to resulting tension in the nerve that may inhibit regeneration. There is a need for improving surgical outcomes and functional recovery.

A potential method to enhance regeneration is through utilizing growth factors. Elucidating the role of pathway-derived growth factors is crucial to promoting regeneration of motor neurons and improving surgical outcomes. A schematic of the post-injury environment for regenerating peripheral nerve can be seen in Figure 1.5. Notably, we see that Schwann cells increase their production of growth factors as a response to injury, and this is thought to play a role in modality-specific regeneration. Regenerating motor axons preferentially reinnervate muscle pathways, indicating the ability of regenerating motor axons to distinguish between motor and sensory nerve [12]. This information may help explain why grafting motor nerve with cutaneous nerve consistently produces worse results than end-to-end nerve repair [13]. While it has been shown that throughout regeneration growth factors can vary as a consequence of peripheral pathway or type of Schwann cell, it is not clear which of these factors are responsible for the modality-specific support of regenerating motor axons in muscle nerve or ventral root. [14, 15]. Growth factor effects are best studied *in vitro*, in a simplified environment in which growth factor specific effects on axons

and cell bodies can be teased out. Thus, the development of a platform that both closely mimics and allows precise control over the post-injury *in vivo* regeneration environment would be very valuable.

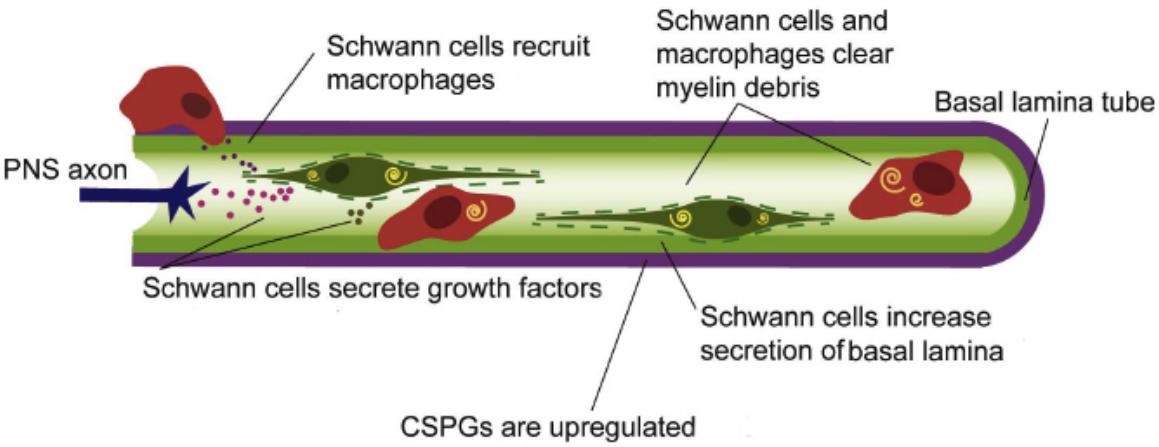

Figure 1.5: The regeneration environment of an injured peripheral nerve can be very complex. Notably, Schwann cells upregulate their production of growth factors, which are thought to play a role in modality-specific regeneration. A method to be able to precisely model the post-injury regeneration environment is thus extremely valuable. This figure was reproduced with permission from [2], with minor modifications.

1.5 Specific Aims

In my dissertation, I present the design and application of novel *in vitro* platforms, both organotypic and cell-culture based, to enable the study of nerve injury and regeneration in locally tailorable environments. This will include microfluidic devices that enable chemical injury and physical injury, and an organotypic device that allows

for both physical and chemical injury in a more three-dimensional environment. My specific aims are:

Aim 1 Determine site of toxicity of paclitaxel-induced degeneration

Aim 2 Determine the site of action of glial cell line-derived neurotrophic factor (GDNF) family ligands in enhancing regrowth after axotomy

Aim 3 Develop a dual chamber organotypic spinal cord and peripheral nerve co-culture device

In further detail:

Specific Aim 1: Determine the site of toxicity of paclitaxel-induced degeneration

Background: Paclitaxel is a commonly used chemotherapeutic agent that has been shown to cause distal polyneuropathies. These symptoms are often a dose-limiting effect of paclitaxel for cancer patients. Although the mechanism is not clear, it is hypothesized that paclitaxel may induce distal axonal degeneration by disrupting microtubule-based axonal transport. Erythropoietin (EPO) is a glycoprotein hormone known to have several different roles in the body, including neuroprotection.

Rationale: Utilizing microfluidic devices, we can elucidate the site of action of paclitaxel, whether cell body or axon, and the most effective site of neuroprotection. If we can determine if paclitaxel is most destructive when applied locally to either the cell body or distal axon, it will help elucidation of a mechanism of action as well as have implications for delivery of chemotherapeutics in order to limit axonal degeneration.

We hypothesize that paclitaxel will be most destructive when applied locally to the axon more so than the soma. If disruption of axonal transport is the likely cause of axon toxicity, local application to distal axons should be most destructive. If EPO's neuroprotective effects can occur through local mechanisms, it would be expected that localized application will be most effective, while if these mechanisms require transport to the cell, application the the soma will be most effective.

Specific Aim 2: Determine the site of action of glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) in enhancing regrowth after axotomy

Background: Many growth factors are upregulated in the distal pathway post-injury. GDNF is one such growth factor that has been shown to promote regeneration both *in vitro* and *in vivo*. The site of action of the regenerative effects of GDNF has not been clearly demonstrated. More so, it would be interesting to determine if other members of the GDNF family of ligands can demonstrate a similar enhancement of regeneration post-injury.

Rationale: By performing axon injury within a microfluidic device, we can investigate whether any regenerative effect of GFLs on sensory axons are due to local or cellular mechanisms. We hypothesize that if these growth factors occur through cellular mechanisms, application to the cell body side would be most effective. This can be further confirmed by concurrent administration of a retrograde transport blocker to determine if growth factor application to the injured axon requires transport back to the cell. In addition, extrapolating GDNF to a tissue-level sensory nerve injury model

may not have similar results to our cell-level study as there are many support cells and extracellular matrix components that may change the situation considerably.

Specific Aim 3: Develop a dual chamber organotypic spinal cord and peripheral nerve co-culture device

Background: There is an unmet need for an organotypic nerve repair model that allows for isolation of the regrowing axon in order to precisely tailor the local environment without disrupting cell bodies. Growth factor effects are best isolated *in vitro*, however, monolayer cell culture techniques do not allow for an accurate model of nerve repair. Organotypic culture allows us to perform experiments at the tissue level where it is possible to recreate a nerve repair environment.

Rationale: A novel device can be designed that includes a spinal cord and peripheral nerve co-culture in order to create a nerve injury platform that allows for independent manipulation of the regenerating axon versus neuronal cell bodies. Injury can be performed by simple transection, while nerve repair can be performed by the addition of new grafts. The device requires viability of cells and axons, as well as demonstration of functional compartmentalization. We hypothesize that such a device can be created using common themes from both microfluidic and Campenot culture systems in a way that ensures viability, repeatability, and compartmentalization.

1.6 Significance and Innovation

The engineering innovation of the proposed project is that this is the development of the first dual chamber organotypic co-culture platform, isolating areas of an embedded membrane, for spinal cord and peripheral nerve co-culture. There is a need for an experimental model that both reproduces the complex three-dimensional environment of peripheral nerve and allows for precise control of the growth factor environment in the distal pathway.

The biological significance is that these studies will contribute to the current knowledge of degenerative chemotherapeutic agents and the role of GFLs in the regeneration of PNS axons, as well as demonstrate the feasibility of utilizing these devices for further pathway-derived growth factor studies on injured neurons. Examining the regenerative effects of pathway-derived growth factors on regenerating motor axons, as well as on healthy, injured, and regenerating sensory axons will help fill gaps in current biological knowledge, and may prove fruitful in the development of therapies for enhancing successful and accurate regeneration post-injury.

1.7 Dissertation Organization

The organization of this thesis is as follows:

Chapter 2 provides a more comprehensive nerve injury neurobiology overview, followed by a discussion on microfuidic devices for studying nerve injury. *Chapters*

3-5 will detail the work for my aims. More specifically, *Chapter 3* presents the determination of the site of toxicity of the chemotherapeautic agent paclitaxel through a microfluidic chemical injury device and demonstrates neuroprotection through the glycoprotein hormone erythropoietin. *Chapter 4* provides a study on the determination of the site of action of GFLs in promoting axonal regeneration through a microfluidic physical injury device and extends that study to the tissue scale. *Chapter 5* discusses the development of a compartmentalized organotypic device for studying nerve regeneration. Finally, *Chapter 6* concludes with a discussion of the significance, major contributions, and future directions.

Chapter 2

Investigation of Nerve Injury through Microfluidic Devices

This review has been published in the Journal of the Royal Society of Interface [16] and reprinted in accordance with the guidelines of the journal.

2.1 Abstract

Traumatic injuries, both in the central nervous system and peripheral nervous system, can potentially lead to irreversible damage resulting in permanent loss of function. Investigating the complex dynamics involved in these processes may elucidate the biological mechanisms of both nerve degeneration and regeneration, and may potentially lead to the development of new therapies for recovery. A scientific

overview on the biological foundations of nerve injury is presented. Differences between nerve regeneration in the central and peripheral nervous systems are discussed. Advances i n microtechnology over the past several years have led to the development of invaluable tools that n ow facilitate investigation of neurobiology at the cellular scale. Microfluidic devices are explored as a means to study nerve injury at the necessary simplification of the cellular level, including those devices aimed at both chemical and physical injury, as well as those that recreate the post-injury environment.

2.2 Introduction

Nerve injuries can often cause devastating functional disabilities. Fortunately, peripheral nerves hold the potential to regenerate after injury, however complete repair and exact functional restorations are not possible. Current state of the art treatment for peripheral nervous system (PNS) injuries involves end-to-end suturing of uninjured nerve ends when the injury is small, and the use of autologous nerve grafts when the injury is large. The use of autologous nerve grafts in clinical peripheral nerve repair is associated with donor site morbidity, the need for multiple surgeries, limited tissue availability, and inadequate functional reinnervation [17–19]. Regeneration is not inherently possible in the central nervous system (CNS) environment, and hence no pharmacological or technological solutions to the CNS repair and regeneration are available [20, 21]. Accordingly, there is a considerable research interest

in studying both nerve injury and regeneration in order to elucidate how to promote successful nerve repair. Investigating nerve injury on a cellular scale offers a unique potential for probing the pathophysiology of injury at the single neural cell level and investigating neural responses to the immediate environment. Traditional *in vitro* cell culture techniques have contributed significantly to our understanding of healthy and diseased neurons [22, 23]. However, these techniques do not provide a controlled environment to grow or guide neurons, or enable precise probing of the cells and evaluation of extracellular or environmental interactions. Modern microfluidic technology offers the potential to accurately model or control the changing neuronal microenvironments. Thus, the precision and control supplied by microfluidic technology may be particularly relevant for the study of nerve degeneration and regeneration.

Neurons naturally operate in the microscale as multistate mechanical, chemical, and electrical sensors and actuators. Their operations occur on a level fundamentally familiar to engineers, and in a way that makes interfacing of neuronal cells with microdevices intuitive [24]. Application of microtechnology or micro electromechanical systems (MEMS), has contributed greatly in the past several years, and offers invaluable tools to facilitate neuroscience studies at the cellular scale. This technology has led to the development of Lab-on-a-Chip (LOC) devices, built using microtechnology, incorporating elements such as microscale channels, pumps, and valves, and offering precise control or manipulation of the neuronal microenvironment in ways previously unachievable with macro-scale methods [5,6,25,26]. In view of their potential, innova-

tive LOC microdevices have been developed to offer a highly structured environment to experiment with neuronal cells. These LOC devices offer spatial control at the level of cell deposition within designed structures, separation of subcellular components through the use of microchannels, and influence over interactions with other cell types [7,24,27–32]. There is also the benefit of precise control over the amount of reagents or factors added to the cells and low cost associated with the devices due to the small volume of expensive reagents required for the devices. There has been some application of microtechnology to *in vivo* implantable devices for nerve regeneration, such as those that incorporate microfluidic chips into nerve implants in order to provide precise delivery of a target drug or enable monitoring of regeneration [33–37]. However, the majority of neuronal LOC devices are *in vitro* devices, in contrast to *in vivo* cell or whole-organism devices, and will be the primary focus of this review.

While studying neurons in their healthy state is undoubtedly valuable, experimentation into the mechanistic understanding of the underlying pathways governing axonal injury and regeneration, a research area of great importance, is less developed and would benefit from advances in the LOC technology [38]. Our intent is to give an overview of the biology of nerve injury and explore microfluidic LOC devices that accurately and selectively injure axons or model the post-injury environment and examine their potential in the field of regenerative neuroscience. Utilization of these types of LOC devices will enable a deeper understanding of axonal injury and regeneration mechanisms, and eventually lead to the development of clinically relevant

therapies.

2.3 Biology of Nerve Injury and Regeneration

The physiology of the nervous system presents distinctive challenges to nerve regeneration. An understanding of the general organization and components of the nervous system is necessary for insights into difficulties that may arise during nerve injury and regeneration. The nervous system is categorized into the CNS and the PNS. The CNS consists of the brain and spinal cord, and serves as the control center, conducting and interpreting signals, while the PNS consists of motor and sensory nerves that transmit signals between the CNS and the rest of the body. The nervous system consists mainly of neurons and glial cells. Neurons, the basic functional units, are made up of a soma (cell body), axons that conduct signals away from the soma, and dendrites that relay signals to the soma. Axons contain the majority of the cells cytoplasm [39]. Glial cells are the support cells of the nervous system, and are much more plentiful than neurons. These cells have some capacity for cell division, unlike neurons which cannot undergo mitosis and proliferation, although they can regenerate or sprout processes under the right conditions [17]. Schwann cells are the glial cells of the PNS, while oligodendrocytes, astrocytes, and microglia are the glial cells of the CNS. Schwann cells form the myelin sheath that insulates peripheral axons. Schwann

cells ensheath axons and the myelin sheath forms concentric layers around the axon, which become tightly apposed. Oligodendrocytes myelinate axons of the CNS, and in contrast to Schwann cells, can myelinate several axons each. Another important distinction is that in the PNS, Schwann cells are surrounded by a neurilemma, which is a basement membrane (basal lamina) similar to the type found in epithelial layers. These characteristic differences can be found summarized in Figure 2.1. The presence of a basal lamina is one of the distinguishing features of the PNS, as CNS axons do not have this continuous basal lamina surrounding their axons [17, 40]. The absence of a basal lamina may contribute to regenerative failure in the CNS, as the basal lamina not only provides access to growth promoting ECM molecules, but may also shield the axons from inhibitory molecules [41].

Upon axonal injury, either through transection or severe compression, axon pathophysiology may proceed through different paths. The axon can undergo a degenerative retraction from the site of injury for a relatively short distance unless the injury is close to the cell body, in which case it proceeds to the cell body where retrograde neuronal degeneration can occur. Otherwise, Wallerian degeneration (WD) of the distal segment occurs, characterized by axonal swelling followed by accumulations of spheroids, and disruption of the cytoskeleton [38]. WD can be described as a series of cellular events that lead to anterograde degeneration of axons and myelin sheaths from the site of lesion to the nerve endings, while the proximal stump undergoes regeneration. A major difference between PNS and CNS neurons is the response to

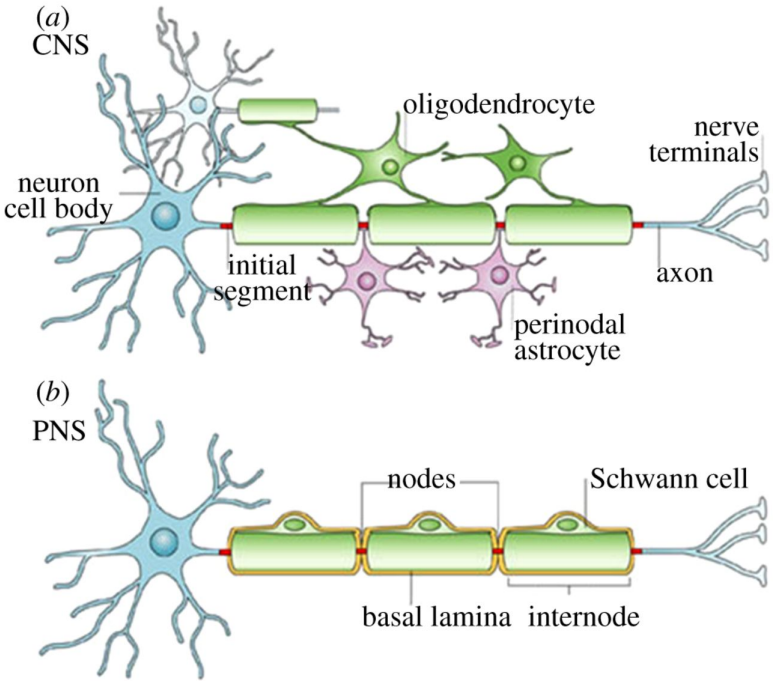

Figure 2.1: A typical CNS neuron is myelinated by oligodendrocytes, with have the capacity to myelinate several cells at once, while a PNS neuron is myelinated by Schwann cells, which myelinate in a one-to-one ratio and are surrounded by basal lamina (reproduced with permission from [40]).

axotomy and injury [17, 38, 42–45]. In the CNS, regeneration does not occur in the native environment, while in the PNS regeneration does occur, although complete functional recovery may not. The inherent ability of PNS neurons to regenerate contrasting with the inability of CNS neurons to regenerate has been a long-standing area of research interest. Injury to the axon reactivates an intrinsic growth capacity in the cell body [46]. In the PNS, in contrast to the CNS, this intrinsic growth capacity is coupled with a locally permissive environment due to a more successful clearance of axonal and myelin debris from the degeneration process by Schwann cells and macrophages, as well as support from axon guidance cues, such as extracellular matrix

(ECM) components, cytokines, and growth factors [47]. Figure 2.2 gives a high-level summary of the differences in CNS and PNS injury and regeneration, demonstrating successful regeneration following injury in the PNS with the aid of Schwann cells, circulating monocytes, and macrophages, contrasted with scar formation in the CNS. The following is meant to be an overview of injury and regeneration in order to provide the rationale and context for the development of neuronal LOC devices; further sources can be consulted for an in-depth biological review [17, 19, 38, 41–50].

2.3.1 PNS Injury and Regeneration

Peripheral nerve injuries (PNIs) can result from stretch-related injuries, lacerations, or compressive trauma [50]. While these types of injuries can all lead to WD, there is typically more preservation of continuity of the neuron with stretch-related and compressive injuries, particularly with regard to the basal lamina, although disruption of this continuity can also be seen in extreme cases [42]. This preservation of continuity of the basal lamina can increase the probability of successful regeneration. Laceration models are seen proportionately more often in the literature due to their ease of reproducibility.

Depending on the extent and site of injury, changes occur at the soma, site of injury, and axon segments proximal and distal to the site of injury. Within hours of injury, axons and myelin start to physically fragment and swell, inhibiting signal

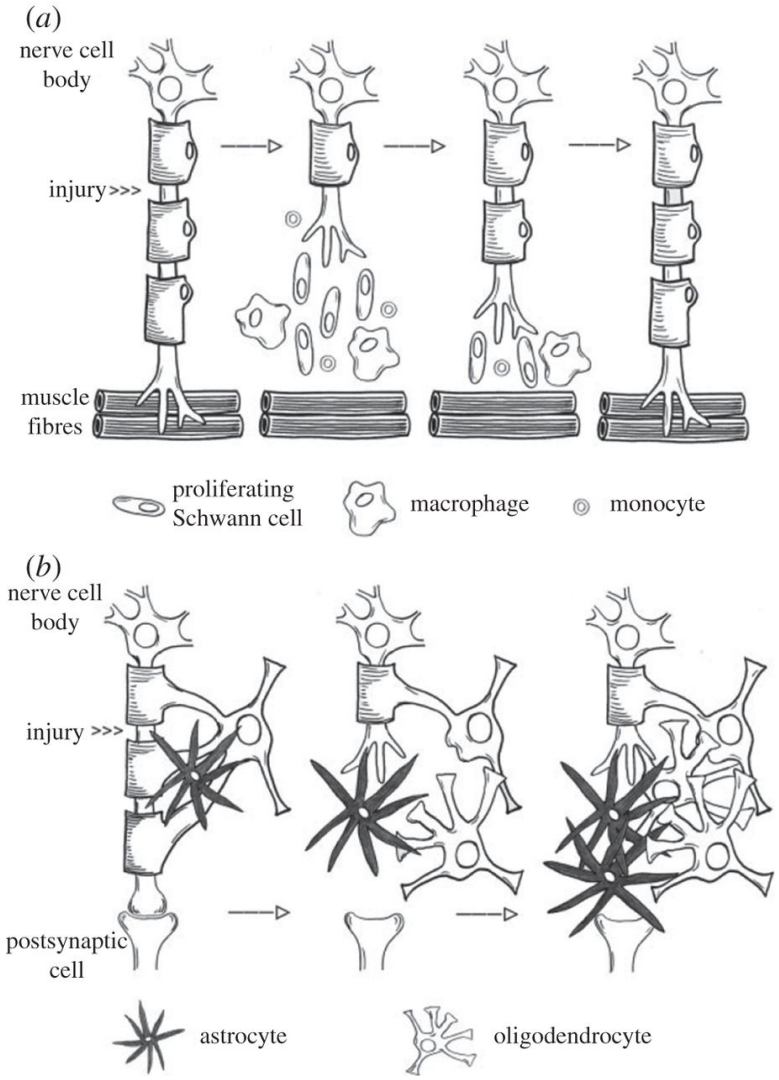

Figure 2.2: A comparison of (a) PNS Regeneration demonstrating a favorable regeneration environment cleared of debris by Schwann cells and macrophages, with (b) the CNS post-injury environment, which includes unsuccessful debris clearance and scar formation (adapted from [50] by [17], reproduced with permission from both).

conduction. In the soma, the nucleus moves towards the periphery of the cell body and there is an increase in cell volume as production of RNA and regenerative proteins increases [49]. Somal proximity to the site of injury and age of the subject determines neuronal survival; the closer the injury is to the cell body and the older the subject,

the more susceptible the neurons will be to apoptosis, with the exception of enhanced sensitivity of neurons to apoptosis in neonates [49, 50]. Similar to WD, the proximal segment undergoes minimal degradation, called traumatic degeneration, depending on the extent of injury. If the injury is sufficiently severe, or proximal to the cell body, this degradation can extend back to the cell body leading to cell death [49]. Schwann cells become active and proliferate, forming dedifferentiated daughter cells that release molecules to help in the degeneration and regeneration process and remove axonal and myelin debris from the site of injury together with macrophages. Distal to the site of injury, focal lesions can trigger WD. These focal lesions need not be transections, but do need to cause a focal block of anterograde axonal transport, such as with a severe compressive injury or transection [44]. Late in the WD process, Schwann cells align themselves in columns along the intact basement membrane, known as the bands of Bunger, serving to guide sprouting axons during regeneration.

The WD process needs to complete before nerve regeneration can occur in severe injuries, however in mild injuries depending on proximity to the cell body, regeneration can begin nearly immediately. Regeneration has been found to be dependent on responses from the cell body, depending on a variety of factors including the age of the subject, severity of lesion, distance from the cell body, location of the injury, and availability of pathway-derived growth factors [51]. The first signs of regrowth may be visible several weeks post-injury for more severe injuries, or as early as 24 hours post-injury for milder injuries [50]. The proximal axon produces multiple sprouts

containing growth cones, normally present during development, that initiate regeneration [38,50]. The reformed growth cones can encounter negative cues such as physical barriers, molecular barriers, and other inhibitory factors. Physical barriers can include glial cells within the site of injury, while molecular barriers include chondroitin sulfate proteoglycans [52]. The regenerating growth cones become misdirected as they attempt to avoid these barriers, and thus become unsuccessful in making connections to target tissues. The growth cone, similar to that in the developmental state, can also be positively guided towards targets through both soluble and bound tropic cues, including growth factors.

The growth cones have an affinity for laminin and fibronectin, ECM components of the basal lamina of the Schwann cell tubes, and use these for guidance. Once contacted, the regenerating axons preferentially grow within these tubes towards the end organ. Schwann cells along the Bands of Bunger also increase production and release of factors such as nerve growth factor (NGF) that act as stimuli for continued axon regrowth and additional guidance cues [50]. Growth factor signaling has traditionally been known to play roles in development, but the role of such signaling has recently been extended to regeneration as well [53]. Far less is known about the role of pathway-derived growth factor signaling in the response of regenerating adult neurons, and the signaling pathways involved in each case may be different [54].

The nature of the site of damage can influence the success of regeneration by affecting growth cones, and can vary based on extent of injury. In a transection there

may be a gap and an extended lesion site, and thus more chances for scarring and space for wandering axons [38]. Outlook for functional recovery after transections that cause a large gap between proximal and distal ends can be negligible. When a nerve repair is performed, post suturing, there can be scarring or poor matching. In contrast, a nerve crush may yield a more favorable setting for regeneration and reinnervation, since the internal structure and surroundings are preserved [38]. Human peripheral axon regeneration rates have been reported to range around 1 mm/day in clinical situations, with further diminishing rates over time [17, 38, 50]. These rates may vary depending on the extent of injury, as well as the type, with higher rates of regeneration in crush injuries and lower rates in transection injuries; regeneration rates expectedly also vary across species. Axonal regeneration does not always mean functional recovery, as misdirection of regenerating axons is common cause of poor functional recovery. *In vitro* studies of peripheral nerve injury and regeneration are valuable in order to elucidate the roles of growth factors in regeneration, as well as to observe how to enhance regeneration rates and functional reinnervation.

2.3.2 CNS Injury and Regeneration

CNS axon injury can result from the mechanical forces associated with the rapid deformation of the brain or spinal cord during trauma. Blunt trauma may cause vascular rupture, decrease the integrity of the blood-brain-barrier, and directly crush nerves or, in the case of severe force, cause complete axotomy [55]. Edema can also

result from trauma, with cytotoxic swelling of neurons, which can in turn cause compression of further tissue. Despite the availability of *in vivo* models, mechanistic understanding of the underlying pathways governing CNS axonal injury remains only partially understood. As regeneration does not occur in the native environment of the CNS, performing injury studies within an *in vitro* platform may help elucidate the mechanisms underlying both axonal injury and regeneration. These types of injuries also share common features and potentially convergent pathways with other conditions that may result in CNS axonal degeneration, including Alzheimers, HIV related dementia, and multiple sclerosis [44]. There is a need for complete understanding of the degeneration process, as severity of axon damage plays a large role in eventual outcomes following degeneration [56, 57].

Focal injuries result from high impact rapid events such as blows to the head, or projectile and penetrating blast injury. Focal injuries can cause hematomas or hemorrhaging that in turn cause further compression [44]. When an object impacts the head, there is an initial focal contact force, and this force may in turn accelerate or decelerate the brain, causing further inertial forces. Focal lesions can trigger WD of distal axons. While dynamic deformation rarely leads to primary axotomy, there does not need to be a complete transection of the axon, as a focal block of axonal transport may be enough to trigger degeneration [44, 58]. Diffuse injuries result from inertial forces and rapid head rotations, as would occur during car accidents and falls [59, 60]. Diffuse axonal injury (DAI) is multifocal, with multiple spheroids appearing

on individual axons, and is characterized by swollen and disconnected axons. The extent of injury is dependent on the mode, severity, duration, and rate of the strain, as well as the distance of the injury from the cell body, anatomical location of the injury, and the mechanical properties of the axon [44, 61]. Axons are viscoelastic and can withstand varying degrees of stretch deformation under normal activities, but are thought to become brittle under dynamic loading conditions, making them vulnerable to damage [58]. The mechanical properties of the axon can also vary with age, previous injuries, and disease. Mechanically stretching cultured axons has been demonstrated to replicate the morphological and structural changes associated with DAI [62].

Damage to the axonal cytoskeleton or a primary axotomy can result from a rapid unidirectional stretch. While it is clear that primary injury results from the direct mechanical strain experienced by cells from injury, the mechanism of the downstream cellular events is not well understood. Changes in molecular gradients occur, as there is sodium influx following injury, which increases swelling and also increases intracellular calcium levels, which may activate proteases for breaking down the cytoskeleton [44, 63]. Axonal transport proteins may accumulate in the areas of swelling due to disruptions in transport, including amyloid precursor protein (APP). Cytoskeletal changes are more apparent upon direct damage to the axolemma, which would occur in severe injury, and it would be beneficial to study these *in vitro*. Recent evidence has also indicated axon-specific degeneration pathways separate from those

related to the cell body, making it beneficial to study axon injury while being able to manipulate the cell body and axon independently [44, 45, 64, 65]. The determination of axon-specific pathways may be important for understanding the cellular and molecular mechanisms for degeneration.

Immediately after injury, CNS axons can display abortive regeneration with an initial outgrowth for up to 0.5 mm, but then come to a stop and die-back and retract or become arrested and form retraction bulbs [38]. It has been demonstrated that CNS neurons can express their intrinsic growth capacity on permissive substrates within the right biological environment, but the growth appears to be too slow for full functional reinnervation [66]. Aguayo initially demonstrated that peripheral nerve grafts can be used to promote CNS axon regeneration [67]. It has also been demonstrated that peripheral nerve grafts are the most promising grafts for CNS nerve repair, promoting regeneration from non-permissive white matter to permissive grey matter in spinal cord repair [68]. The natural CNS environment is regarded as unfavorable with inhibitory effects from glial scars, chondroitin sulfate proteoglycans (CSPGs), and myelin-associated proteins, inadequate inflammatory responses, and hindered debris clearance due to the presence of the blood-brain-barrier. Glial scars formed at the site of an injury act as both mechanical and biochemical barriers for regrowing axons. As the name suggests, the scar is often comprised of glial cell types including reactive astrocytes, microglia, oligodendrocyte precursors, and fibroblasts. It also contains growth inhibitory factors such as semaphorins, nephrins, tenascin and

chondroitin sulfate proteoglycans [61, 69, 70]. Myelin-associated inhibitors found in the glial scar, such as Nogo-66, myelin-associated glycoprotein, and oligodendroctye-associated glycoprotein, have been shown to interact with the Nogo receptor (Ngr) to inhibit neurite outgrowth [71]. In contrast, extracellular matrix component molecules such as laminin have been found to promote regeneration [51]. Although the CNS contains microglia, they do not aid in debris clearance to the extent of Schwann cells, which attract macrophages to enhance clearance, as well as produce neurotrophic and neurotropic factors to aid in regeneration [55]. The availability of a model that allows for the study of CNS regeneration and determines the effects of these factors within an injury platform would be valuable.

2.4 Microfluidic Devices for Studying Nerve Injury and Regeneration

Traumatic injuries, both in CNS and PNS, can lead to irreversible damage resulting in permanent loss of function. Studying the complex dynamics involved in these processes may elucidate the biological mechanisms of nerve regeneration and degeneration, potentially leading to the development of new strategies and therapies for nerve regeneration and recovery. *In vivo* animal models of trauma have permitted the study of a multitude of complex variables and permit the analysis of behavioural outcome, enabling monitoring of prognosis and determination of functional outcome to various

treatment strategies. These *in vivo* animal experiments include models of instant rotational injury, impact acceleration injury, lateral fluid percussion injury, controlled cortical impact, spinal cord compression and contusion, nerve stretch, and complete nerve transection, as previously reviewed [72, 73]. These models have provided much insight, but they are highly complex, containing multiple parameters, have potentially low reproducibility, and are time consuming and labor-intensive. More importantly, they do not allow for monitoring axonal regeneration in real-time, or enable study at the reductionist single cell level necessary for determining precise mechanisms. In contrast, as an alternative parallel experimental system, *in vitro* models allow the study of biochemical pathways, gene expression levels, and phenotypic changes at the level of a single axon, which are extremely relevant in the study of traumatic axonal injuries.

Microfabrication technologies enable the development of powerful platforms to grow and manipulate neurons and in order to model and study axon injuries. These LOC devices can be made using modified semiconductor fabrication technologies, including photolithography, etching, and deposition methods, in order to construct microscopic structures in glass, silicon or polymeric materials such as poly(dimethylsiloxane) (PDMS). These platforms can play a role in the development of novel and innovative surgical and repair strategies for damaged PNS axons. Conventional mass produced metal surgical instruments are affordable, but are not durable and degrade quickly, while instruments made of diamond and ceramics are durable but are expensive.

MEMS-based technology can enable development of unprecedented Si probes and blades with enhanced performance characteristics and cost of production. For cell culture, LOC devices provide perfusion to cells or enable probing of cells and cell compartments with chemical reagents. Microfluidic platforms further facilitate study at the single cell resolution of axons isolated from soma through compartmentalization. Composite microfluidic platforms, with compartments for neurons and means to carry out highly parallel experiments constitute high-throughput LOC devices. Such devices enable precise control over cellular microenvironments, require small volumes of reagents, and have potential for automation and multiplexing [29, 74]. Microfluidic systems have been broadly employed for neuron cell culture, neuron manipulation, neural stem cell differentiation, neuropharmacology, neuroelectrophysiology and neuron biosensors [28, 75].

The most well-known microfluidic devices for neuronal study are compartmentalized LOC systems fabricated through PDMS soft lithography. There are several properties that make PDMS an excellent choice for biological studies. As a material, PDMS is inexpensive, flexible, and easily fabricated and bonded to other materials. It is also biologically inert, nontoxic to cells, impermeable to water, permeable to gases, and optically transparent down to 230 nm, facilitating microscopy [76]. Typically, a master wafer is created using standard photolithography techniques and replicas created from the mold using PDMS soft lithography [5]. Fabrication of these types of devices has been covered elsewhere, and will not be the focus of this review [5, 25, 77].

LOC platforms facilitating compartmentalization and thus separation of axons from cell body allows precise tailoring and manipulation of the microenvironment of axons, distinct from the cell body, as is the case in many *in vivo* situations. Compartmentalization enables more in depth studies of myelination, neurite outgrowth, drug screening and protection, signalling, as well as the study of networks ranging from cellular to organ levels in organotypic cultures [5, 26, 78].

Microfluidic platforms are highly compatible with incorporation of different injury modalities. Incorporation of an injury platform within a microfluidic culture system would allow for better determination of axon-specific mechanisms in degeneration and regeneration by allowing for independent manipulation of axon and cell body. In addition, locations of injuries respective to cell bodies can be generalized within a range for arrays of axons. Microfluidic platforms can be broadly classified as devices that model chemical and physical injury, and devices that model the regeneration environment. Here we provide an overview of exemplary injury devices.

2.4.1 Microfluidic Chemical Injury Devices

The simplest microfluidic injury devices provide chemical injury. Traumatic injuries to axons of CNS and PNS can be induced by chemicals such as chemotherapeutics, neurotransmitters in excess (excitotoxicity), and detergents [8, 79, 80]. One of the first and most cited microfluidic LOC device designed to study neurons in their various compartment structures (soma, axon, dendrites) was the device created by

Jeons group [5]. Fluidic isolation of axonal chamber from somal chamber is achieved through microliter level volume differences between the two chambers. Microchannels between the chambers provide high fluidic resistance that leads to a small but sustained flow that counteracts diffusion. This device can be seen in Figure 2.3. Potential neurotoxins can be localized precisely in the axonal compartment. Several studies have utilized this type of device and its derivatives for examining axon injury and regeneration [6, 29, 32, 81]. These types of devices also include the ability to direct sites of neuronal attachment and neurite outgrowth through micropatterning techniques. Hosmane et al. created a multiplexed circular version of the platform which utilized centrifugation to enhance axonal throughput through microchannels, and demonstrated increased microglial accumulation to aid in debris clearance near the site of injured CNS axons seen in Figure 2.4 [82]. Peyrin et al. developed a three compartment microfluidic device to study simultaneous axonal degeneration and death mechanisms of CNS axons subject to axotomy with precise spatiotemporal control [79]. The injury was induced by a brief and isolated flux of detergent in the central compartment. In their proof of concept for the device, they observed rapid Wallerian-like degeneration in the distal axons subject to axotomy, consistent with *in vivo* axotomy. Li et al. developed an integrated microfulidic platform to chemically induce axonal injury and study the recovery and regeneration of axon either in co-culture with glial cells in controllable chamber using valves or treatment with monosialoganglioside, a drug aiding neuronal regeneration [80]. Their results

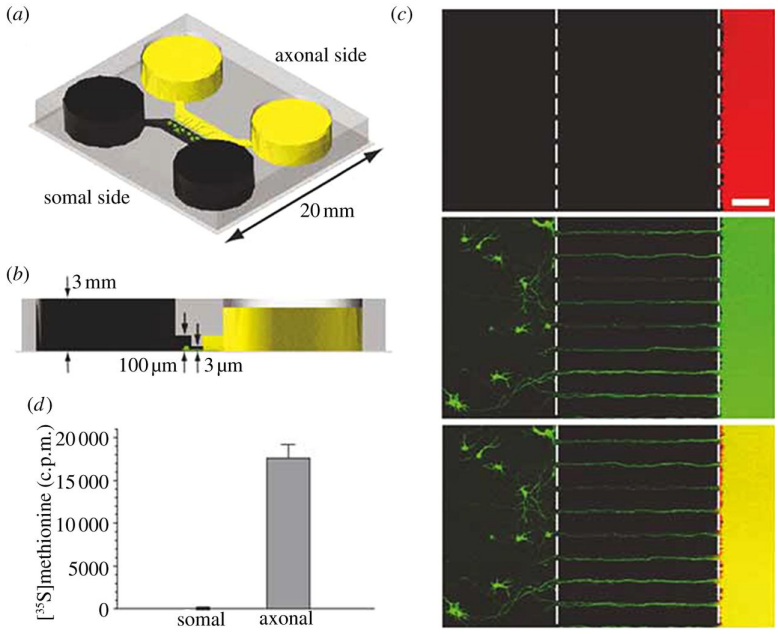

Figure 2.3: A microfluidic compartment device (a) with volume differences between the two chambers (b) to maintain isolation across microchannels. Isolation was demonstrated (c) by localization of texas red and (d) radioactive S35methionine (reproduced with permission from [6]).

indicated that axons were more resistant to injury upon localized application of acrylamide compared to soma, and that axons had self-destruct programs different from soma, where injury to the soma caused secondary axon collapse.

2.4.2 Microfluidic Physical Injury Devices

Physical modes include employing aspiration, physical cutting, laser ablation techniques, valve based compression of axons [83–86]. Microfluidic platforms have been utilized as valuable tools to study axon regeneration *in vivo*. Many model organisms such as Aplysia californica, Caenorhabditis elegans, Drosophila and zebrafish

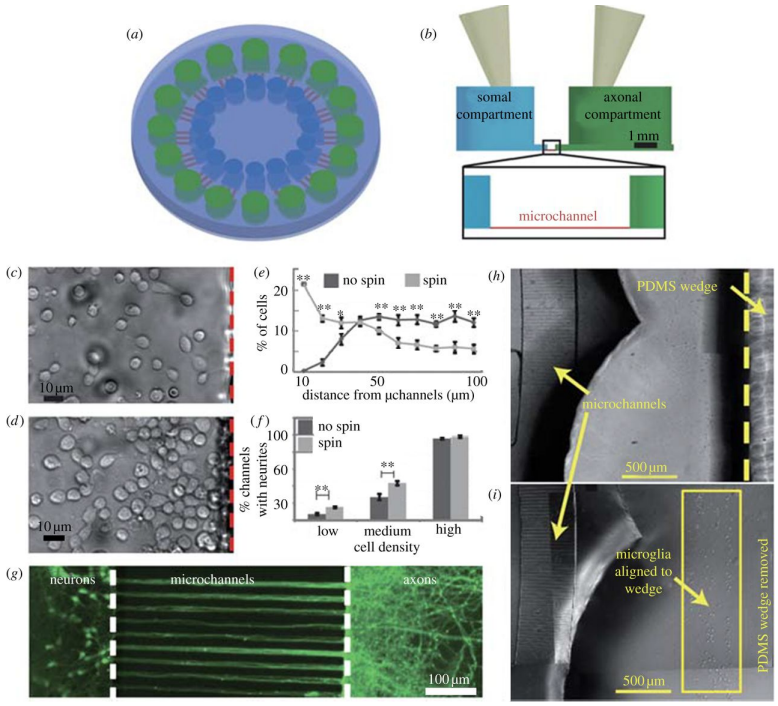

Figure 2.4: A circular microfluidic platform demonstrating (a) enhanced axonal throughput through spinning, (b) multiplexing capacity, and (c) utilization of stencils to localize microglia (reproduced from [71] with permission from the Royal Society of Chemistry).

have been used for *in vivo* neuron injury and regeneration studies [80, 83–85, 87–94]. C.elegans in particular provides an interesting paradigm for studying nerve injury and regeneration as its genome has been completely sequenced, and *in vivo* axotomy for the organism is feasible. The critical step of immobilizing the worm and subjecting it to axotomy has conventionally been done through the use of glue and anesthetics. These methods can either have unknown toxic effects that are difficult to evaluate or are labor intensive and of low throughput. Microfluidic platforms can provide a clever alternative to these techniques. The immobilization can be achieved in several ways like anesthetizing, cooling, or trapping the worm using deflectable valves [26,95].

Choksi has developed microfluidic platforms to immobilize single worms on either a short term or long term basis to characterize their on-chip behavior [84]. The immobilization is achieved in one of the two approaches; either CO_2 is utilized to change the micro-environment and cease the worms movement in a long term fashion, or a deformable membrane is utilized to mechanically restrict the worm. A behavior module revitalizes the worm after immobilization through mechanical stimulation, consisting of a saw shaped microchannel that forces the worm to move in a sinusoidal pattern so that its locomotion can be analyzed.

While studying nervous system injury in model organisms such as C. elegans, an enormous volume of screening studies often needs to be done involving massive image acquisition and processing, data acquisition and interpretation. MEMS platforms can be integrated with imaging to increase the performance and throughput of these studies. The potential for automation is high on these platforms because of their small size and scale. A robust and high throughput performance can be achieved on these platforms [96]. Chung designed and developed an automated, integrated microfluidic system to perform high-throughput microsurgery [85]. This device is capable of processing multiple worms in parallel without increase in control complexity. The device can be used to simultaneously load worms in one set of channels and perform imaging and laser ablation in the other set. Guo developed a high throughput microfluidic platform for *in vivo* nerve regeneration studies that enables precise focusing and nanosurgery of trapped worms and feeding for recovery of the operated worms

in two different modules [83]. The device also incorporates an adjustable trap for immobilization of worms at various developmental stages. Highly specific laser ablation techniques can be used to injure the worms once they are steadily immobilized. Using this chip, they observed faster than expected rates of axonal regeneration, and that distal fragments of the severed C. elegans axon regrow in the absence of anesthetics. Based on the frequency or the repetition rate of laser, the gaps created in the axotomy vary. With high frequency lasers 2-5 m gaps are created where as low frequency lasers result in precise 1-2 m gaps [87, 97]. Other physical modes of injury to axons involve needles to transect individual axons, fluid percussion, and microelectrodes [79].

The mechanical and cellular response to injury can be quite complex, but study of such stimuli can be simplified by using controlled cellular injury *in vitro* models. These models include several advantages over *in vivo* animal models including the ability to monitor real-time acute injury responses [61]. Existing *in vitro* injury models have subjected neural cultures or explants to the forces experienced during traumatic CNS injury, and include stretchable deformable membranes, two-photon laser ablation, and hydrodynamic shear based axotomy through microfluidic channels [79, 98–100]. Recent advances in culturing neurons within hydrogels has allowed for the development of three dimensional cultures that allow for bulk deformation [101].

Mechanotransduction is the study of cellular adaptation to internal and external mechanical stress. Cells elicit a downstream biochemical signal in response to variations in forces acting on a cell. Several tools have been developed to study the

combinatorial interplay between mechanical properties and force induced biochemical changes in cells [102]. The mechanotransduction of CNS and PNS injuries provide powerful insights for development of treatment strategies, and accordingly, several *in vitro* platforms have been developed to study mechanotransduction in injury. Stretch induced injury is one of the many modes of mechanotransduction and physical injury observed *in vivo* during traumatic insult to axons of CNS and PNS. Several groups have modelled and studied these injuries *in vitro*. A stretch-induced injury model of rat cortical astrocytes was developed by culturing the cells on a deformable membrane which was subjected to deformation by a positive rapid pressure [103]. The astrocytes were grown in tissue culture wells on flexible silastic bottoms to which a pressure was applied that stretched the membrane and in turn the astrocytes in order to study the morphologic, physiologic and biochemical consequences of stretch-induced injury. The system enabled the study of the extent and degree of injury with precise control over membrane deformation by varying the amplitude and duration of pressure. Cell injury was demonstrated to be proportional to the degree of silastic membrane deformation, with increasing stretch causing mitochondrial swelling, disruption of glial filaments, and vacuolization. This is one of the earliest *in vitro* attempts to study injuries in cells derived from the brain. With the advancement in MEMS, novel platforms could be developed to study the role of mechanotransduction. in traumatic axonal injuries. The forced being applied on an axon determines its fate of degeneration, regeneration, or stalling in place.

In a more recent study, Hosmane developed a valve-based axon injury platform that enabled compression of CNS axons at the micron-scale, seen in Figure 2.5 [86]. The valves controlled push-down pads that descended upon application of compressed gas. The pressure of the gas was modulated in order to create different levels of injury. Increased pressures, more likely to cause axotomy, were found to promote subsequent axonal regrowth. In another example, Smith applied continuous mechanical tension to axons, and achieved a sustained, rapid growth [104]. The device physically split integrated neuronal cultures into two halves and separated the halves progressively further apart using a microstepper motor system. In doing so they achieved a growth rate of 1mm/day. Transecting axons to induce axonal injury by laser ablation, as discussed previously in whole organisms, is another physical injury technique [81, 97, 100, 105]. Kim et al. developed a neuro-optial microfluidic platform that integrates a microfluidic chip, femtosecond laser for axotomy and mini-incubator to maintain a sterile and appropriate microenvironment for long term monitoring of events post-injury [100]. An example of the laser ablation achieved within these devices can be seen in Figure 2.6.

These injury devices also contained soluble and surface bound inhibitors within the injury compartment in order to better mimic the regeneration environment *in vivo*. Sretavan et al. developed a microdevice to assist the axon regeneration after injury [21]. This device included the development of a silicon nitride knife with ultra-sharp knife edge with a 20 nm radius of curvature produced utilizing MEMS technology.

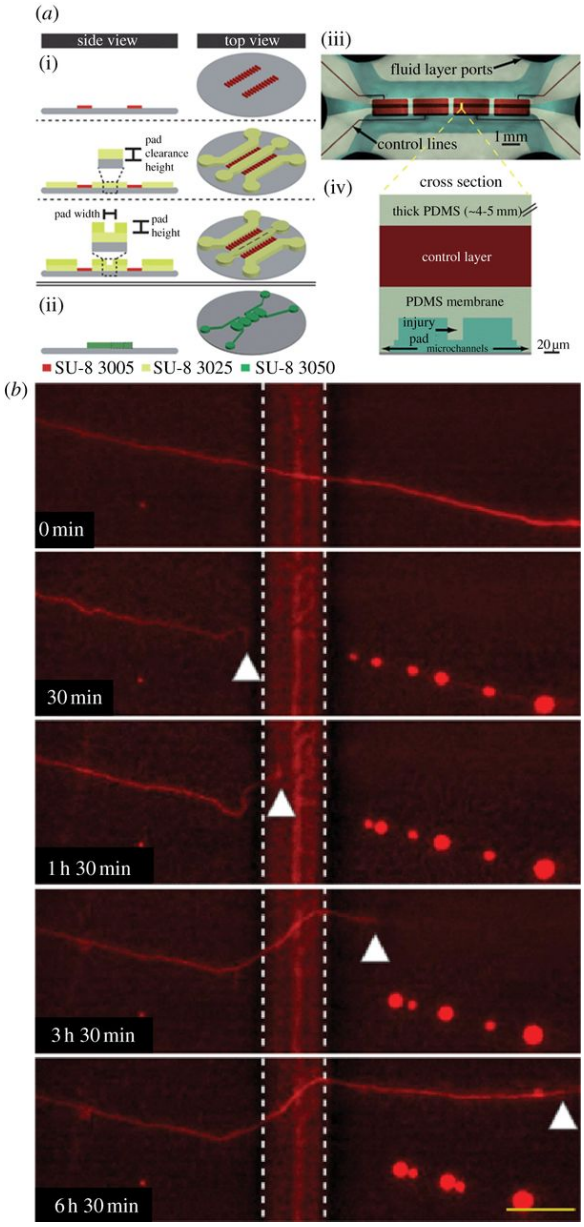

Figure 2.5: (a) A valve-based injury platform with images detailing the (i) cell layer process flow and (ii) control layer process flow, followed by (iii) top down and (iv) cross-section images to visualize control layer (red) versus cell layer (blue). (b) Below, axon compression, degeneration, and regrowth are monitored real-time within a representative device, with degeneration apparent at an image from 30 minutes, and the start of regrowth apparent in the 1 hour and 30 minute image (reproduced from [86] with permission from the Royal Society of Chemistry).

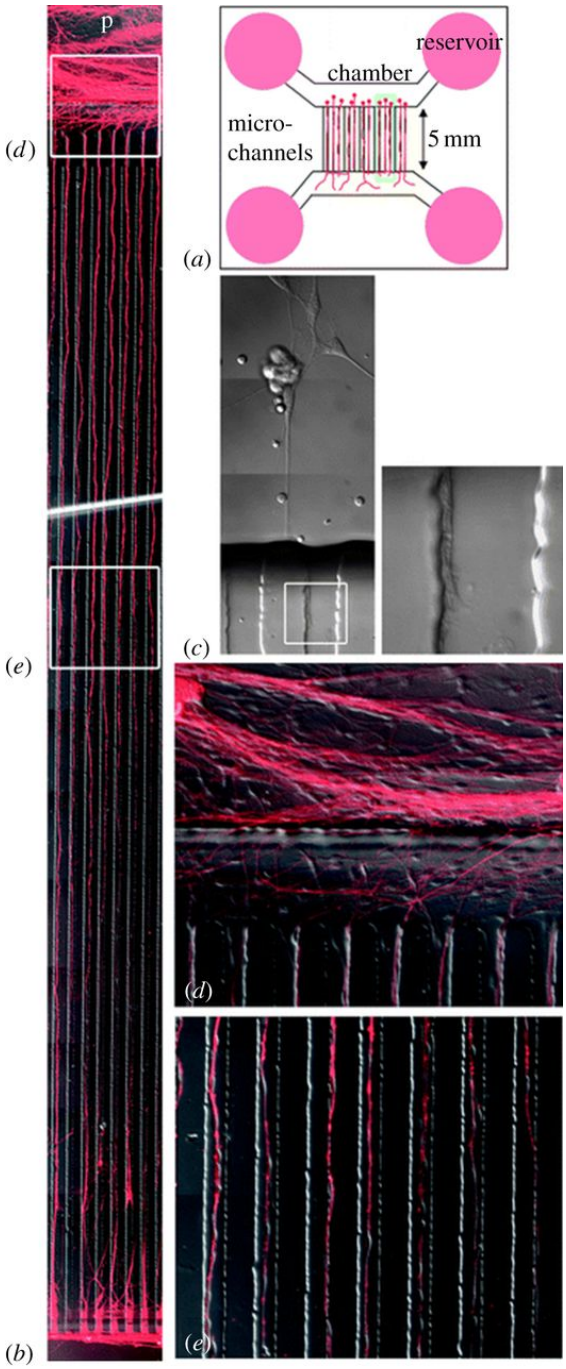

Figure 2.6: (a) Schematic of the laser-based injury device can be seen, along with (b) representative images of DRG growth through microchannels, and a higher magnification of the same. (c) A representative axon bundle cut with a femtosecond laser demonstrates high thermal confinement of the site of injury. Axons can be seen (d) entering and (e) traveling through microchannels in higher resolution images (adapted from [100] with permission from the Royal Society of Chemistry).

This knife was used for cutting regions of damaged axons individually. These damaged segments were replaced with healthy donor axon segments through dielectrophoresis (DEP), which was used to manipulate and line up the donor axon segments in the region of interest. The axon membranes of the segments were successfully electrofused.

2.4.3 Microfluidic Devices Recreating Regeneration Environments

One of the major obstacles for regeneration is the distance or gap between the proximal extending axon and the distal stump. In bridging this gap, axons have to circumvent the non-permissive substrates for neurite growth, while certain growth factors and other transient molecules may aid positive guidance. Recent studies have showed that targeting a specific group of extracellular inhibitory factors in itself was insufficient to promote long-distance regeneration of CNS axons. Hur et. al aimed to promote regeneration by directly targeting the growth cone through pharmacological inhibition or genetic silencing of nonmuscle myosin II (NMII) [78]. As part of this study, the axonal compartment was coated with inhibitory chondroitin sulphate proteoglycans, and the effect of applying blebbistatin, a specific inhibitor of NMII ATPase activity, was examined. As can be seen in Figure 2.7, application of blebbistatin allows axons to overcome inhibitory cues. The inhibition of NMII causes reorganization of microtubules and actin in the growth cone in a way that allows for

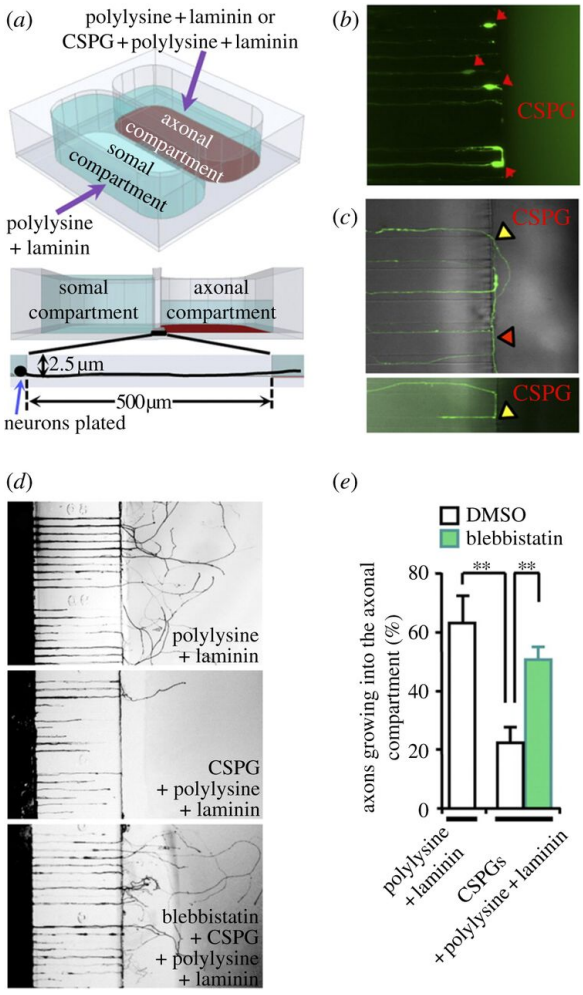

Figure 2.7: Inhibition of NM II allows axons to overcome inhibitory substrates in the axonal compartment of a microfluidic device. (a) Schematic of the two-compartment chamber is given. (b,c) Representative images of DRG neurons facing the inhibitory chamber demonstrate inability to breach the border. (d,e) Blebbistatin was locally applied to the axonal side, demonstrating an ability to overcome inhibitory cues (reproduced with permission [78]).

rapid axon extension, both over permissive and inhibitory substrates.

Over the time course post-injury, chemical gradients of several neurotrophic factors are established; these gradients may significantly influence the growth dynamics of axons post-injury and may even play a role in determining the fate of axons towards regeneration or degeneration. Establishing gradients in neuronal culture may deliver powerful insights about regrowth dynamics. MEMS platforms, due to their operating dimensions and small scale, make it feasible to establish stable gradients over time. For example, gradients can be established on these platforms by exploiting the surface tension differences between two ports connected by a microchannel. This same principle is applied to develop and demonstrate a passive pump in microfluidic devices [106].

LOC devices that mimic the repulsive or attractive chemical cues present in the regeneration environment provide insight into how to best guide regrowing axons towards appropriate targets. In the event of injury, neurites need to perform the non-trivial tasks of reorganizing and re-establishing existing connections. Kothapalli et. al developed a novel microfluidic device to study neurite guidance under the influence of chemo-gradients [107]. The neurons were cultured in micro channels on a physiological 3D environment of collagen type-I, and gradients were established of chemo-attractants such as Netrin-1 and chemo repellents such as Slit-2. The gradients developed were stable up to 48 hours. This time frame allowed for the qualitative and quantitative study of neurite turning, providing valuable insight into the development,

maintenance and reorganization of complex neural networks. The ability to monitor and guide regrowing axons can enhance our efforts in promoting functional recovery.

2.5 Conclusions

Nerve injury is a widely observed, but difficult to study phenomenon, particularly *in vivo*. The current standard treatment for peripheral nerve injury, such as end-to-end surgical reconnection, or reconnection with an autologous nerve graft, are also highly limited. While in the periphery nerve regeneration occurs, functional recovery may not. In the central nervous system, the outlook is starker, as no treatments are currently available. A more complete understanding of the neurobiology of nerve injury and regeneration in both of these systems may improve surgical or biomaterial or scaffold-based repair outcomes and functional recovery. Therefore, *in vitro* methods are of interest so as to very precisely and microscopically observe nerve injury and develop and test different repair strategies. Continuing advances in the field of microtechnology enable the creation of devices capable of studying regeneration at the reductionist cellular scale, allowing for the ability to tease out mechanisms that may be lost at the complex *in vivo* setting. Chemical injury can be easily achieved within a microfluidic platform, while physical injury is accomplished through the incorporation of other technologies such as lasers, nanoscale ultra-sharp knives, and valve-based compression. The post regeneration environment can also be modelled,

through chemical modifications of the device surface or alteration of fluid flows. While the strength of microfluidic devices are in their reductionist, highly controllable environments, currently available microfluidic devices do not perfectly mimic the *in vivo* environment, particularly as most operate at the two-dimension level and many effects may not translate from the cell level to *in vivo*. Large surface to volume ratios and particularly miniscule amounts of media may lead to cell viability issues due to evaporation and potential difficulty in maintaining cell culture conditions, if not tightly managed. The devices have promoted the formation of interdisciplinary and collaborative research teams, but wide-scale adoption by neuroscientists, although expected, has not yet occurred. Despite these concerns, microfluidic devices continue to look promising for investigating axonal regeneration. A variety of LOC devices discussed in this review point to a vibrant field where novel platform technologies are facilitating cellular discoveries and basic research is promoting the development of novel platform technologies. Next generation devices are expected to better mimic the three-dimensional *in vivo* regeneration environment, as well as incorporate other advances in other fields such as optogenetics and biosensors, in order to extend fundamental findings from cellular studies and take a step closer to realizing clinical therapies for enhancing nerve regeneration in both the PNS and the CNS.

Chapter 3

Compartmentalized microuidic culture platform to study mechanism of paclitaxel-induced axonal degeneration

This chapter has been published as a paper in Experimental Neurology [8] and reprinted in accordance with the guidelines of the journal.

3.1 Abstract

Chemotherapy induced peripheral neuropathy is a common and dose-limiting side effect of anticancer drugs. Studies aimed at understanding the underlying mechanism of neurotoxicity of chemotherapeutic drugs have been hampered by lack of suitable culture systems that can differentiate between neuronal cell body, axon or associated glial cells. Here, we have developed an *in vitro* compartmentalized microfluidic culture system to examine the site of toxicity of chemotherapeutic drugs. To test the culture platform, we used paclitaxel, a widely used anticancer drug for breast cancer, because it causes sensory polyneuropathy in a large proportion of patients and there is no effective treatment. In previous *in vitro* studies, paclitaxel induced distal axonal degeneration but it was unclear if this was due to direct toxicity on the axon or a consequence of toxicity on the neuronal cell body. Using microfluidic channels that allow compartmentalized culturing of neurons and axons, we demonstrate that the axons are much more susceptible to toxic effects of paclitaxel. When paclitaxel was applied to the axonal side, there was clear degeneration of axons; but when paclitaxel was applied to the soma side, there was no change in axon length. Furthermore, we show that recombinant human erythropoietin, which had been shown to be neuroprotective against paclitaxel neurotoxicity, provides neuroprotection whether it is applied to the cell body or the axons directly. This observation has implications for development of neuroprotective drugs for chemotherapy induced peripheral neuropathies as dorsal root ganglia do not possess bloodnerve-barrier, eliminating one of the cardinal

requirements of drug development for the nervous system. This compartmentalized microfluidic culture system can be used for studies aimed at understanding axon degeneration, neuroprotection and development of the nervous system.

3.2 Introduction

Peripheral neuropathy is a relatively common disease that affects at least 2.4% of the population in the US [108]. Prevalence increases with aging population and reaches as high as 26% of people older than 65 [109]. Typical symptoms of peripheral neuropathy reflect sensory, motor, or autonomic nerve fiber dysfunction. In particular, sensory symptoms include paresthesias, some of which are painful, sensory loss and numbness. In most polyneuropathies, these symptoms begin distally in extremities and progress proximally [110, 111]. The pathologic changes in most of these polyneuropathies are those of a distal to proximal axonal degeneration, which have been referred to as dying-back neuropathies [112]. Currently there are no effective therapies aimed at the underlying mechanism of axonal degeneration, except for inflammatory neuropathies characterized by infiltration of peripheral nerves with lymphocytes and macrophages [113].

Paclitaxel, a diterpene alkaloid drug, is a commonly used chemo-therapeutic agent against breast, lung and ovarian cancer. One of the major dose-limiting side effects is distal axonal, mainly sensory, polyneuropathy [114,115]. The symptoms of paclitaxel-

induced neuropathy are tingling, numbness, loss of balance and burning pain. The mechanisms of neurotoxicity of paclitaxel are largely unknown and effective treatments for axonal neuropathy caused by paclitaxel are currently not available. Paclitaxel binds to beta-tubulin and stabilizes its polymerization. This leads to disruption of the mitotic spindle and arrest of cell division [116, 117]. It has been suggested that paclitaxel may lead to an increase and altered distribution of detyrosinated tubulin, a marker for stable microtubules [118]. In addition to these *in vitro* studies, animal models of paclitaxel neuropathy have been developed in rodents [115, 119–123], but the underlying mechanism of distal axonal degeneration induced by paclitaxel remains to be determined.

Although multiple *in vitro* models of peripheral neuropathies exist only two groups have attempted to use compartmentalized culture systems to ask whether axonal degeneration is due to local axonal disturbances or a consequence of derangement in the neuronal cell body [120, 121, 124]. These groups used Campenot chambers that consist of a Teflon divider attached to collagen-coated Petri dish with silicone grease [4]. Campenot chambers require great skill, as leakage between chambers is a common problem, limiting efficiency and reproducibility. Chamber systems, other than Campenot chambers, have been developed to isolate hippocampal [125] and motor axons [126] from soma using thin coverslips. However, these had similar problems as the Campenot chambers, leakage between chambers being the most common one. In contrast, advances in microtechnology and biomaterials have led to numerous

approaches that precisely control the positioning of cells on substrates [127–130]. Presenting cells with controlled topographical and chemical cues have allowed us to understand a great deal about how cells respond to their local microenvironments [131–134]. Microfabrication technology, utilizing photolithography, micro-contact printing, and microfluidics, is used to construct chambers in which precise *in vitro* cellular patterning is achieved. Such microfabrication techniques have been used to create chemical and biochemical analysis platforms [128, 135, 136].

In this study, we aim to help elucidate the underlying mechanism of paclitaxel-induced axonal degeneration through the use of microfluidic platforms that allow us to physically and fluidically isolate cellular compartments, as well as to gauge the protective role of recombinant human erythropoietin. Probing different cellular compartments allows us to determine whether the site of action is on the cell body or axonal side. Since most polyneuropathies are dying- back neuropathies, we sought to determine if paclitaxel caused the most degeneration when applied to the distal axon as compared to the cell body. Erythropoietin is a glycoprotein hormone that has effects on multiple organs and tissues. We have previously shown that this hormone is involved in an endogenous neuroprotective pathway through Schwann cell-derived erythropoietin [137], and demonstrated this effect both *in vitro* and *in vivo* [120, 121]. The mechanism of action is not precisely known, and thus we wish to determine if there is a differential effect based on application of the hormone to cell body or axonal side for its implications in the treatment for polyneuropathies. We used microfabrica-

tion techniques to develop a novel microfluidic platform to efficiently load and isolate neuronal cell bodies from axons with independent manipulation of the neuronal cell body and axons. Using this platform we demonstrate that the chemotherapeutic drug paclitaxel is toxic at clinically relevant pharmacological doses when applied to the axons but not to neuronal cell bodies. Furthermore, we show that recombinant human erythropoietin can protect against this axonal toxicity even when it is applied to the neuronal cell body compartment.

3.3 Materials and Methods

3.3.1 Microfabricated Chamber Preparation

A two-step photolithographic process was utilized to create the master mold as depicted in the schematic in Figure 3.1. Silicon wafers (University Wafer, MA) were coated with SU-8 2002 (Microchem; MA), spun, and soft baked using parameters specified by the manu- facturer to yield a resist thickness of 2.5 m. An array of microchannels (Figure 3.1A), each with dimensions: width = 10 m, length = 500 m, were defined by UV light exposure through a high resolution DPI transparency (Cad/Art, OR). The exposed substrate was once again baked, to enhance polymer cross-linking post exposure, and developed as stated in the resist technical sheet to fully define the microchannels. The process was immediately repeated with SU-8 3050

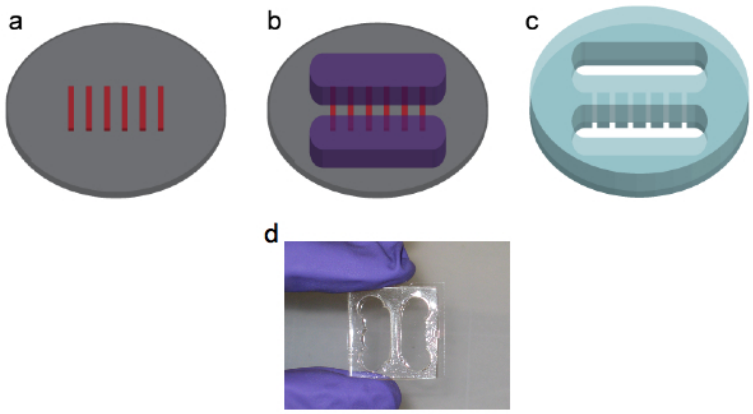

Figure 3.1: The device mold was constructed using standard SU-8 photolithography. (a) The first resist layer (h = 2.5 μm) defined an array of microchannels while the (b) subsequent step (h = 150 μm) defined larger fluidic ports and reservoirs (not drawn to scale). (c) Silicone rubber was then poured and cured over the mold to yield the final device structure. (not drawn to scale). A picture of the final device can be seen in (d).

(Microchem; MA) to define the fluidic reservoirs with dimensions: width = 3 mm, length = 13 mm (Figure 3.1B). The master mold was then treated with trichlorosilane (United Chemical Technologies; PA) for 30 min to create a nonstick surface for subsequent processing. Standard soft lithography was performed using Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning, MI) as described previously [138]. After curing, the PDMS was carefully removed from the master and access ports were created using a suite of dermal biopsy punch tools (3-6 mm) (Huot Instruments, WI) (Figure 3.1C).

3.3.2 Cell Preparation

All experiments involving animals were conducted according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Unless otherwise noted tissue culture supplies were obtained from Invitrogen (Carlsbed, CA). Dorsal root ganglia (DRG) neuronal cultures were prepared as previously described [139]. Briefly, DRGs were dissected from decapitated embryonic age day 15 rats. Once obtained, cells were enzymatically dissociated with 0.25% Trypsin in L15 medium and then suspended in media. The DRGs were maintained in Neurobasal medium containing 10% fetal bovine serum, 20% glucose, 1% penicillin/streptomycin, B-27 supplement, 2 M L-glutamine, and 10 ng/ml glial derived nerve growth factor (GDNF). Two days after seeding cells, neurobasal media containing 10 μM of cytosine arabinoside was added to the cultures in order to decrease the amount of glial cells. Paclitaxel (Sigma-Aldrich) was dissolved in cremophor EL/ethanol (50/50 v/v) for a stock concentration of 5.0 mg/ml and stored at -20°C. Recombinant human erythropoietin (EPO) was obtained from R&D Systems (Minneapolis, MN) and dissolved in phosphate buffered saline (PBS) and stored at 20°C.

DRG neurons were loaded into the soma side of devices and grown for 5-7 days to allow axons sufficient time to grow through channels and into the axonal side at a sufficient length. Paclitaxel was diluted in neurobasal media to achieve a concentration of 25 ng/mL and applied to either the neuronal cell body or axonal side. EPO was diluted in culture medium and applied to neuronal cell body or axonal side. Cells

were subsequently stained with calcein AM (green) at a concentration of 2.5 μM for 1 h and imaged using a fluorescent microscope.

3.4 Results

In order to identify the susceptibility of axons and cell bodies to paclitaxel, we added 25 ng/mL of paclitaxel to either axon or cell body chambers and continued to culture the DRGs for another 24 h. Once images of the fluorescently labeled cells and axons were captured, we used ImageJ to calculate axon lengths and calculated percent change in axon length compared to 24 h before taxol exposure. In Figure 3.2, we see images of the DRGs before and after paclitaxel exposure, all taken at the same magnification. In these images, we see the axons exiting channels on the left side and going into the axonal compartment on the right. Figure 3.2 A and B show axons before and after paclitaxel was administered to the axonal compartment, respectively. We can see a noticeable difference in the axon length, as well as morphology. Figure 3.2 C and D show axons before and after paclitaxel was applied to the neuronal cell body compartment. There is not as noticeable a difference in the axon morphology or length. From these images we see that paclitaxel caused axonal degeneration when applied to the axonal compartment, but not when applied only to the cell body compartment.

Studies on the neuroprotective effect of EPO were performed with concurrent

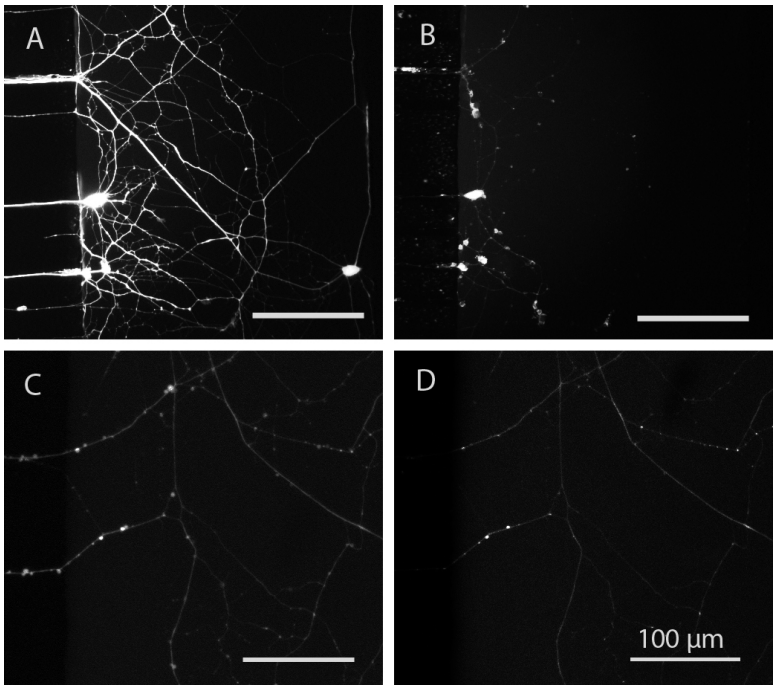

Figure 3.2: Paclitaxel induced local axonal degeneration. Axonal degeneration upon axonal administration of paclitaxel for 24 h is seen only with local axonal application (A = before paclitaxel; B = after paclitaxel) but not with paclitaxel application to the neuronal cell body chamber (C = before paclitaxel; D = after paclitaxel).

administration of paclitaxel, which was applied to either the neuronal cell body or axonal side. In order to study the effect of EPO on different cellular compartments, we also applied the hormone to either the neuronal cell body or axonal side of the chambers. Figure 3.3 A and B show axons before and after administration of EPO and paclitaxel to the axonal side of the chamber, respectively. From these images we see that there is not a large difference in axon morphology or length, demonstrating the neuroprotective effect of EPO as it seems to prevent axon degeneration. This effect was also seen when EPO was applied to the neuronal cell body compartment when paclitaxel was applied to the axonal compartment. Quantification of the results can

be seen in Figure 3.4. Axon degeneration was defined as the change in axon length over the total axon length expressed as a percentage. When only paclitaxel was applied to the axonal side, we saw a 28.6+11.2% decrease in the length of axons, compared to a control in which no paclitaxel was added that showed a 0.42 + 0.25% increase in axon length. Paclitaxel applied in combination with EPO on the axon side showed a 1.02 + 0.42% decrease in axon length, which was more comparable to the control than to the paclitaxel-induced degeneration condition. When paclitaxel was applied on the axon side while EPO was applied to the soma side, we saw a 1.34 + 0.59% decrease in axon length, again demonstrating a neuroprotective effect.

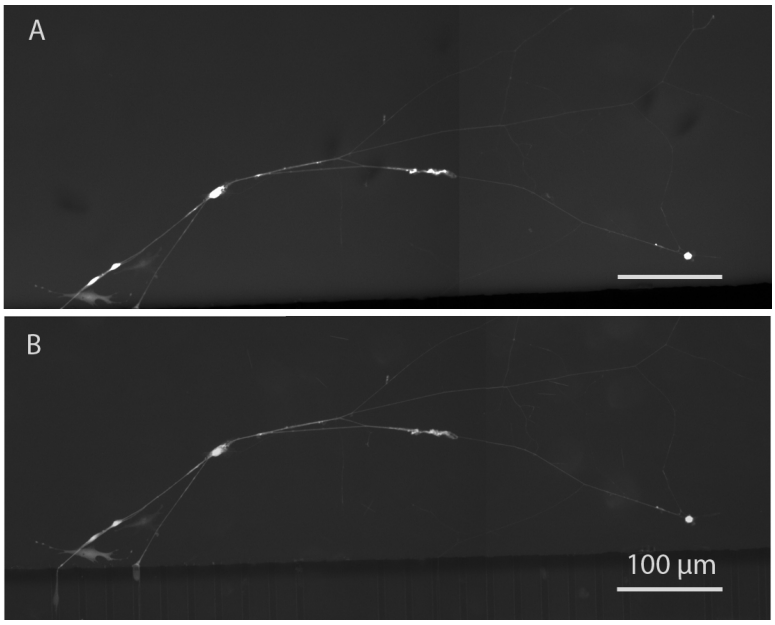

Figure 3.3: Neuroprotective effect of EPO. A protective effect of EPO is observed 24 h after concurrent administration with paclitaxel to the axon compartment (A = before paclitaxel+EPO; B = after paclitaxel+EPO).

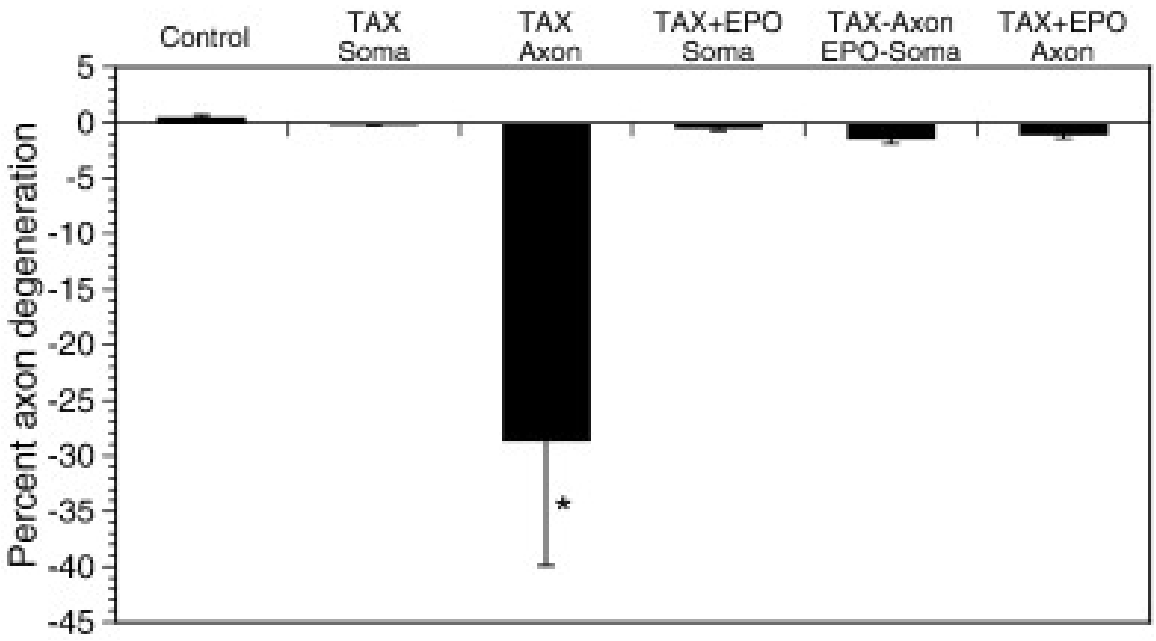

Figure 3.4: Quantification of axon degeneration with paclitaxel administration and axon protection by EPO. Axon lengths were measured on the axonal chamber before and 24 h after application of paclitaxel with or without EPO and expressed as a percent change from baseline. Average N=10 for conditions. ($*$ denotes $p < 0.05$)

3.5 Discussion

Paclitaxel-induced sensory neuropathy is a frequent and disabling side effect, and can potentially lead to the discontinuation of chemotherapy. The microfluidic platform used in this study allowed us to better clarify the mechanism for paclitaxel-induced degenera- tion. The device used in this study does not allow mixture of culture fluids between chambers and provide glass substrate for better optical microscopy compared to Campenot chambers and its derivatives. Furthermore, the microfluidic platform is based on the PDMS, which has excellent gas exchange properties. Recently, Taylor and colleagues developed a microfluidic chamber for the

compart-mentalized culture of neurons [5]. However, these chambers had difficulties in cell loading close to microchannels and low number of axons per microchannel. In contrast, the microfluidic platforms used in this study are simple to load with sufficiently high-density cells and corresponding higher amounts of axons inside microchannels. There was ample segregation of neuronal cell bodies and axons.

In a previous study, we had shown that paclitaxel can cause axonal degeneration but not neuronal death at pharmacological doses used in chemotherapy [120, 121]. What we did not know at that time was whether this axon degeneration was due to local toxicity of paclitaxel in the axon or a consequence of disruption of cellular events within the neuronal cell body. This study clearly shows that paclitaxel causes axon degeneration through local mechanisms, but that this local toxicity can be controlled by intracellular events induced at the cell body as shown by the EPO data. We know that paclitaxel induces an increase in detyrosinated tubulin, thereby leading to cold-stable microtubule assembly within the axons [116, 120, 121, 140]. How this leads to axonal degeneration is still unknown, although prevailing hypothesis is that it interferes with axonal transport depriving distal axons of their vital nutrients and cellular substrates. Rapid degeneration seen in previous studies and in our culture system suggests perhaps a different mechanism. A curious observation we had in our axonal chambers was presence of axonal blebbing even in the most proximal segments of axons in paclitaxel-treated axonal chambers (data not shown). Axonal blebbing is often regarded as a prelude to axonal degeneration but it can be a reversible process

[141]. Local axonal activation of protein degradation pathways, such as caspases or calpains could lead to axonal blebbing and eventual degeneration.

We used the microfluidic chamber platform to examine the site of axon protective action of EPO, expecting that local axonal application of EPO would prevent axonal degeneration induced by paclitaxel. We, however, found that EPO was able to prevent paclitaxel-axonal degeneration even when it was applied to the cell body, away from the axon and paclitaxel. Although we do not know the mechanism of this axon protection, it is possible that EPO–induced changes in intracellular signaling events are transported down the axon using fast axonal transport and block the toxicity of paclitaxel. We do not know if this type of potential mechanism of neuroprotection may apply to other axon protective therapies, but if it does, then the implications for drug development for peripheral neuropathies are immense. One of the limitations of developing therapies for nervous system indications is that axons and neurons are behind a blood-brain/nerve-barrier. This requires that the drugs be able to cross the blood-brain–barrier. However, there are exceptions to this rule and the blood-brain–barrier within the dorsal root ganglia is very leaky. If axon protection can be achieved by action of a drug on the neuronal cell body, even for toxins that cause local axonal degeneration, there would be a less stringent requirement for the drug to cross the blood-brain–barrier. Future studies will help us define if this is a general principle.

In summary, we have developed a novel microfabricated platform, composed of a microfluidic culture system, that is robust, easy to manufacture and reliable. It

allows separation of axons from neuronal cell bodies and independent manipulation of each compartment. It can be used to study mechanisms of axonal degeneration, protection against axonal degeneration and developmental events such as myelination. Furthermore, the manufacturing process is scalable to generate templates with more than hundred chambers that can be independently manipulated, thus allowing high-content studies including drug screening. Through the use of this device, we have demonstrated that paclitaxel causes degeneration of axons through local mechanisms. We have also shown that this effect can be counteracted through the administration of EPO both at the cell body and at the axon, indicating exciting implications for drug development for polyneuropathies.

Chapter 4

Compartmentalized microfluidic culture platform to study axonal regeneration and localized effects of GDNF

4.1 Abstract

In this chapter, the characteristics of compartmentalized microfluidic platforforms are further investigated and the devices are utilized in a physical axotomy model. The regenerative effects of members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) is then explored post-axotomy. As discussed

previously, microfluidic compartmentalized devices contain high fluidic resistance microchannels where hydrostatic pressure is utilized to create a small but sustained flow to counteract any diffusion. We examined this property further by characterizing the diffusion profiles of molecules in the device both theoretically and experimentally. A fluidic height difference of 2 mm was required to localize fluorescent molecules in one side of the compartment. This height difference was used to determine the diffusion profile of a representative growth factor used for subsequent experiments. For these experiments, the effects of members of the GFLs, specifically neublastin, neurturin, and GDNF, were investigated in order to compare their ability to regenerate the axons of dorsal root ganglion (DRG) neurons after *in vitro* axotomy within the microfluidic chamber. The DRGs were grown in the cellular compartment and axons were allowed sufficient time to grow through a microchannel array and into a neighboring axonal compartment where they were severed by gently scratching with either a glass pipette or metal syringe tip. GDNF, neuturin, or neublastin were administered to the axonal or cell body compartments to enhance axonal regeneration. GDNF was most potent in promoting axon outgrowth after axotomy, although all explored growth factors demonstrated some effect. Application of GDNF to either cell body or axon side was effective in enhancing regeneration. To investigate this further, we performed experiments within microfluidic cultures with concurrent application of GDNF with a retrograde transport blocker, cytochalasin D. The initial experiments demonstrated that GDNF applied to the axon is no longer as effective, indicating the importance

of transport to the cell body. To examine the effect of GDNF at the tissue level, we explored the application of GDNF to adult DRG explant cultures, both with and without the presence of laminin. Results indicate the effect of GDNF is strongest during the initial days following injury and application, and that this effect is most pronounced without laminin. These factors can be considered for eventual growth factor based treatments for the enhancement of sensory axon regeneration.

4.1.1 Chapter Organization

This chapter is organized slightly differently than other chapters in this dissertation. After an introductory overview, this chapter presents the characterization of fluidic isolation in microfluidic devices, including theoretical and simulated predictions followed by experimental verification. Compared to the paclitaxel experiments in chapter 3, these experiments are longer in duration, and hence the characterization of fluidic isolation was essential. After this section follows the growth factor experiments section, including the materials, methods, and results at both the cellular and tissue level. The discussion concludes, covering all sections.

4.2 Introduction

Compartmentalized cell culture devices have become widely used in neuroscience studies. As discussed in Chapter 2, while the Campenot chamber is the prototypical

device of this type, more recently, microfabricated devices have been developed to separate neuronal cellular components with enhanced control. In particular, polydimethylsilosane (PDMS) based microfluidic devices have been extensively used in isolating axons of both CNS and PNS neurons [8,82,129]. Specifically, PDMS enables high fidelity molding of microscale features, is biologically inert, optically transparent, and contains tunable mechanical properties. These types of devices have been discussed in detail in Chapter 2. These devices allow for physical separation of cell body from soma through size limitations of the microchannels, which are only large enough to allow axons to pass through, as well as fluidically isolated both due to a combination of the high resistance of the microchannels and an applied hydrostatic pressure [142, 143].

In this study, we sought to characterize fluidic isolation of somal and axonal compartments and examined the site of action of three neurotrophic factors in an *in vitro* axotomy model of sensory axonal regeneration. To characterize fluidic isolation, we examined and verified parameters essential to growth factor diffusion. We determined the diffusion profile of growth factor within our devices to confirm small molecule isolation through theoretical, computational, and experimental means. Once characterized, we proceeded with the growth factor experiments. We examined three members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs) as they play important roles in neural development and different neurological diseases [144–149]. These growth factors have been shown to promote survival and

differentiation of dopaminergic neurons, motor neurons and sensory neurons from dorsal root ganglia (DRG) [150–155]. GDNF in particular has been investigated due to its therapeutic potential. Previous studies have evaluated the role of GDNF in the nervous system in settings of disease and traumatic injury [156–158]. In addition to GDNF, other members include neurturin, neublastin (also known as artemin), and persephin [159]. It has been demonstrated in both clinical trials and animal experiments that GDNF enhances myelination [139,160,161]. GDNF also enhances survival of motor neurons in models of amyotrophic lateral sclerosis [162–164], and dopaminergic neurons in models of Parkinson's disease [165, 166]. Similarly, neurturin has been shown to increase survival of motor and dopaminergic neurons [167–169] and neublastin was effective in reducing neuropathic pain [170–172]. While GDNF has shown great therapeutic potential, the biological site of action of GFLs in axon regeneration is not well characterized.

Identification of biochemical mechanisms involved in axonal injury can be difficult in a complex *in vivo* experimental setup. In addition, standard cell culture does not allow for the compartmentalization of axons from neuronal cell bodies, thus making it difficult to delineate axon-specific mechanisms. Using a microfluidic chamber system we examined the regenerative potential of GDNF, neurturin and neublastin. From previous demonstrations that GDNF is upregulated post-injury and has regenerative potential, we expect GDNF and related GFLs to enhance axon outgrowth post axotomy. If this effect is truly primarily based on cellular versus local mechanisms at

the site of injury, we expect the concurrent axonal application of the growth factor with cytochalasin D, a retrograde transport blocker, to be less effective than axonal application of the growth factor alone.

Due to several environmental differences *in vitro* versus *in vivo*, including the enhanced presence of endogenous growth factors as well as the presence of more substantial support cells and ECM components, we expect that the *in vitro* and *in vivo* results may not be identical. In order to investigate the role of GDNF at the tissue level, we performed experiments with adult DRG explants. Laminin is prevalent throughout the body within ECM and is known to have axonal growth promoting properties both *in vivo* and *in vitro*, and thus experiments were performed with and without the presence of laminin [51, 173]. We expect the results help to further elucidate GDNF's role in regeneration following injury as well allow for further factors for consideration in its potential use as a therapeutic treatment.

4.3 Characterization of Growth Factor Diffusion

Prior to GFL experiments, the fluidic isolation in our microfluidic devices was characterized by several methods due to the longer duration of experiments as compared to the previous chapter. First, a theoretical profile of growth factor diffusion was developed incorporating the experimental parameters of our system. Next, com-

putational simulations were performed to further characterize and visualize diffusion within the devices. Finally, experimental verification of small molecule isolation was performed by observing the diffusion of a comparably small fluorescent molecule over time.

4.3.1 Theoretical Profile of Growth Factor Diffusion

In order to identify whether growth factors can diffuse from the axonal to cell body compartment during the treatment of growth factors in the axonal compartment, we developed models to simulate this experimental setup. The central idea behind the compartmentalized platform is to have fluidic isolation between compartments facilitated by high resistance microchannels. If the microchannels contain a small cross-sectional area ($< 30\mu m^2$), this device paradigm allows axons to grow from one compartment into another but attenuates the diffusion of molecules from the compartment of lower hydrostatic pressure to the compartment of higher. In our experiment, a small differential pressure gradient was established with a higher pressure in the axonal compartment as compared to the cell body compartment. As a result, a low velocity retrograde flow was created in the microchannels to prevent molecular anterograde diffusion. We show theoretically that chemical isolations are achieved when working with the aforementioned parameters. First we formulate the

diffusion-advection problem (Figure 4.1).

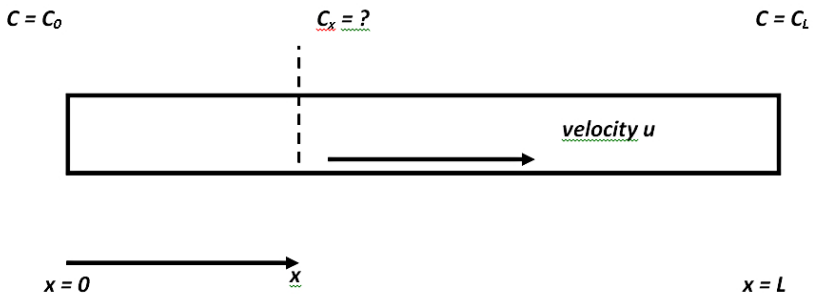

Figure 4.1: Formulation of the diffusion-advection problem

The diffusion of any molecule in a fluid medium is governed by the diffusion-convection equation. The first term including spatial derivatives of concentration describes passive diffusion while the second term including the velocity of the medium describes active diffusion (or the convective element):

$$\frac{\partial C}{\partial t} = D \cdot \nabla^2 C - \overrightarrow{v} \cdot \nabla C \tag{4.1}$$

Where C is the concentration at a point (x,y,z) at time t, D is the coefficient of diffusion, $\overrightarrow{v}$ indent is the velocity vector at the point (x,y,z) at time t. The pressure and concentration gradients driving the dynamics are mainly along the groove (x-axis) and hence, this can be approximated as a one-dimensional problem. Steady state is achieved when

$$D\frac{d^2 C}{dx^2} = u\frac{dC}{dx} \tag{4.2}$$

where u is the x-component of the velocity. Solving this with appropriate boundary conditions yields the following solution:

$$C_x = C_L \cdot e^{-\frac{u}{D}(L-x)} \ and \ C_0 = C_L \cdot e^{-\frac{uL}{D}} \tag{4.3}$$

For pressure (gravity) driven flows, C_0 can be expressed as

$$C_0 = C_L \cdot e^{-\frac{\rho g \cdot \Delta h \cdot a^2}{16 \eta D}} \tag{4.4}$$

For the purpose of simplifying calculations, media is approximated as water. In this equation, Δh is the height difference of the water column at inlet and outlet that leads to the pressure difference which drives flow, ρ is the density and η is the viscosity of water (saline) and a is the channel height (the most critical dimension for laminar flow). Due to the geometry of the system, Δh is limited to approximately 2 mm, and a is limited to 2.5 μm, which is the height of the groove. The other constants used are g=9.81 m/s^2, η=0.00089 m^2/s, and ρ=1000 kg/m^3.

The diffusion coefficient D is calculated indirectly from its inverse dependence on the square root of molecular weight of the diffusing species. D_{oxygen} is known to be 2 x 10^{-9} m^2/s. The molecular weight of oxygen (M_{oxygen}) is 16 Daltons.

$$D_{growthfactor} = D_{oxygen} \cdot \sqrt{\frac{M_{oxygen}}{M_{growthfactor}}} \tag{4.5}$$

The smallest molecule we use is 4.5 kDa, which also thus has the highest tendency to diffuse. It is sufficient to use this molecule for our calculations as the amount of

Table 4.1: Growth Factor Molecular Weights and Calculated Diffusion Coefficients

Growth Factor	Molecular Weight (kDa)	Diffusion Coefficient (m^2/s)
Human GDNF	21 (cited from [174])	5.5 x 10^{-11}
Rat Neurturin	19.5 (cited from [168])	5.7 x 10^{-11}
Rat Neublastin	4.5 (cited from [175])	1.2 x 10^{-10}

GDNF diffusing to the somatic compartment will also be less than the amount of rat neublastin diffusing to the somatic compartment. So, considering the diffusion of rat neublastin:

$$\frac{C_0}{C_L} = e^{\frac{1000\text{x}9.81\text{x}0.002\text{x}(2.5\text{x}10^{-6})^2}{16\text{x}0.00089\text{x}1.2\text{x}10^{-10}}} \tag{4.6}$$

Thus, there is negligible diffusion from the axonal compartment to the somatic compartment at steady state. Care must be taken that while performing experiments utilizing these culture systems that the height difference in the two compartments must be achieved before adding the growth factor to the axonal compartment so that there is always an anterograde flow preventing diffusion of species in the retrograde direction. This height difference must be maintained throughout the experiment.

4.3.2 Computational Simulations to Study Diffusion Patterns of Growth Factors

Simulations were done in Comsol Multiphysics (Formerly FEMLAB; Comsol Inc., MA), a finite element-modeling package. The geometry was simplified to study the diffusion pattern in only one of the microchannel grooves. Each microchannel has a plane of symmetry passing though the middle (a plane going from floor to ceiling all along the length halfway between the two vertical walls). Further simplification of the geometry was performed using such symmetric considerations, so that only half of a microchannel needed to be simulated (Figure 4.2. Simulation consisted to meshing the architecture into a grid of smaller elements. The aspect ratio of such finite elements was tailored to suit the aspect ratio of the microchannel, that is, elements were longer along the length (x-axis) than along the width or height (y and z axes respectively). The geometry was first solved for fluidic parameters such as velocity and pressures at all points. This was done by solving the continuity and Navier-Stokes equations for the microchannel. While all three cases were simulated as described above, we present the results of rat neublastin (the smallest molecule we used) diffusion in Figure 4.3. This suffices to demonstrate the paucity of small molecule diffusion through a microchannel feature.

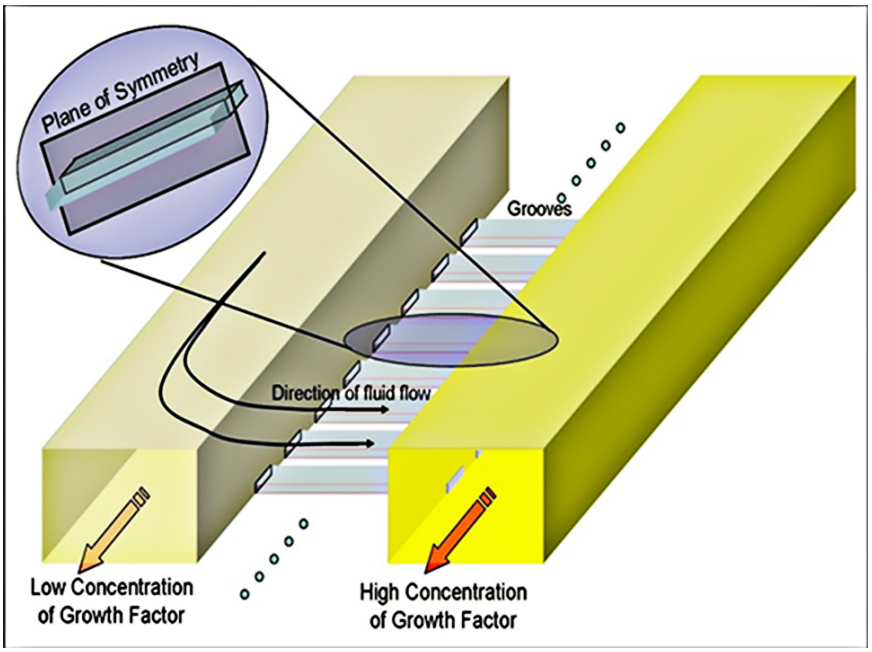

Figure 4.2: Handling of microchannel geometry for computational simulations. Half the microchannel groove is meshed for computational solutions of fluid dynamics and diffusion-convection equations.

4.3.3 Restriction of Fluorescent Molecules

In order to confirm our theoretical and simulated findings of small molecule diffusion within our microfluidic chambers, experimental verification was performed. A hydrostatic pressure was established between the compartments by establishing fluid volumes such that the somal compartment was of lower fluidic height than the axonal compartment. This hydrostatic pressure created a small flow to counteract diffusion. A 1 microliter bolus of fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) dye was introduced to the somal compartment and was imaged over the course of 24 hours. Empirically, a fluid height difference $\geq$ 2 mm was sufficient to prevent a low-molecular weight compound (700 Da) from diffusing from the axonal compart-

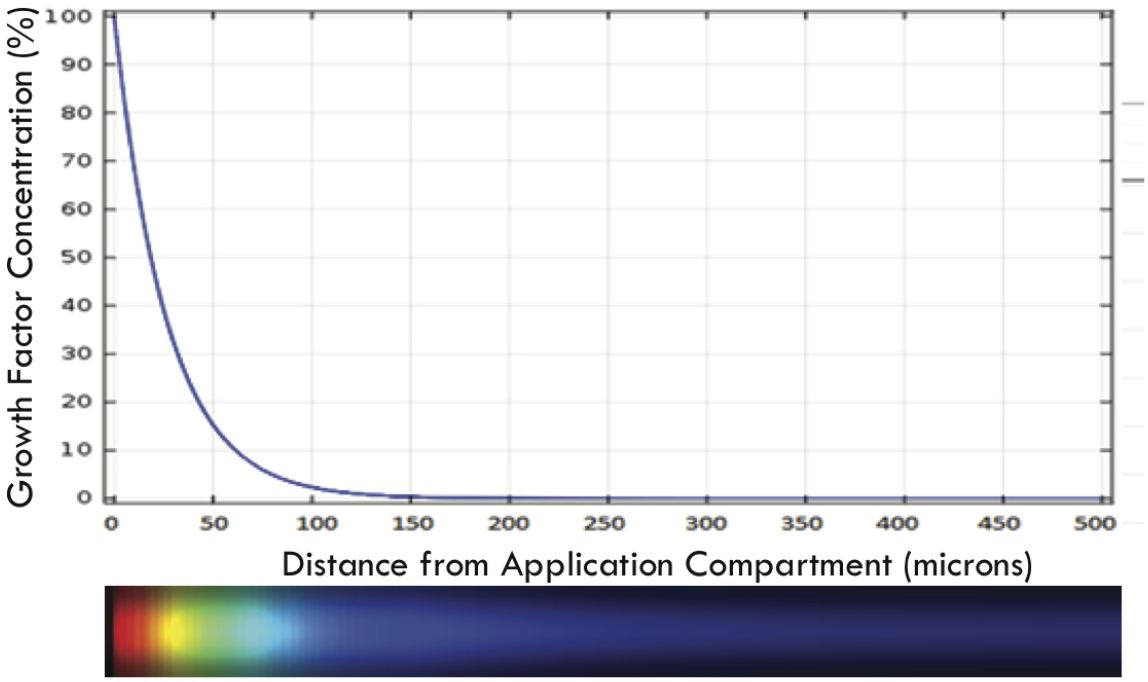

Figure 4.3: Computational simulation of the diffusion profile of growth factor along a microchannel groove. The plot demonstrates that when growth factor is applied to the axonal side, the concentration along the groove drops to negligible amounts within approximately 100 μm in a 500 μm channel.

ment to the somal compartment, which matches both our theoretical and simulated conclusions. In Figure 4.4, we see that under these conditions, the diffusion of the dye was approximately 100 μm after 24 hours, well under the channel length and very close to our prediction. This height difference was maintained by adding 5 μL of media to the higher volume compartment daily, allowing for localization of an applied compound for longer periods. After verifying growth factor isolation theoretically, in a simulation, and experimentally, we proceeded with the experiments to investigate the effect of GFLs on axonal regrowth following axotomy.

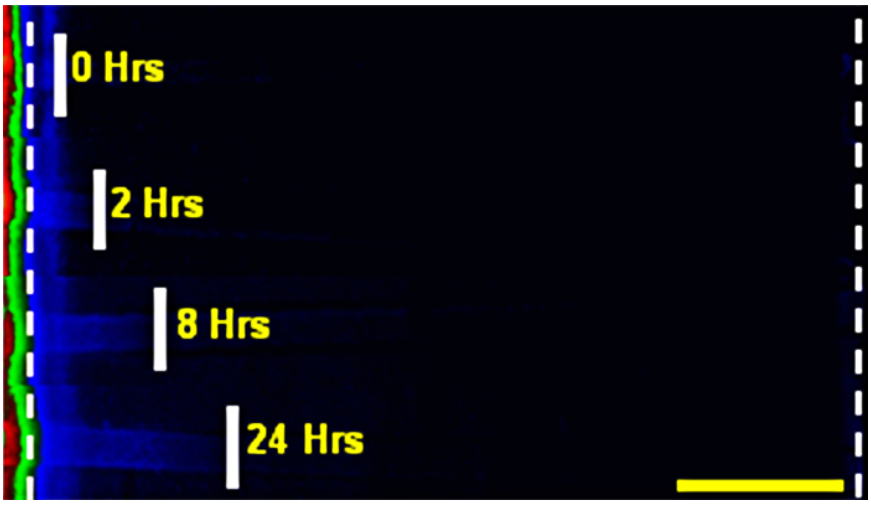

Figure 4.4: Analyte restriction maintained for 24 hrs. Diffusion of a small (MW 700) fluorescent analytes were examined under high hydrostatic pressures. Microchannels (region between dashed lines) connect compartments of unequal fluid height. Establishment of fluid heights >2 mm prevented entry of dye (solid white lines) into the compartment of higher fluid height. Scale bar 100 μm.

4.4 Materials and Methods

4.4.1 Cell and Adult DRG preparation

All experiments involving animals were conducted according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Unless otherwise noted tissue culture supplies were obtained from Invitrogen (Carlsbed, CA). DRG neuronal cultures were prepared as previously described [139]. Briefly, DRGs were dissected from decapitated embryonic age day 15 rats. Once obtained, cells were enzymatically dissociated with 0.25% Trypsin in L15 medium and then suspended in media. The DRG neurons were maintained in Neurobasal medium containing 10% fetal bovine serum, 20% glucose, 1% peni-

cillin/streptomycin, B-27 supplement, 2 M L-glutamine, and 10 ng/ml GDNF. One day after seeding cells, neurobasal media containing anti-mitotic agents (either 10 μM of cytosine arabinoside, or a combination of 50 μM each of uridine and 5-fluoro-2'-deoxyuridine) was added to the cultures in order to arrest the division of glial cells, and then removed the following day.

DRG explants were harvested from sacrificed adult Rosa mice expressing red fluorescent protein in sensory neurons, ranging from spinal levels L3-L6. For a subset of experiments, spinal origin level was noted, with a focus on L4-L6. Once explants were obtained, they were placed in dissection medium, containing Hanks Balanced salt solution (HBSS) (Gibco, Grand Island, NY), 4.3 mM sodium bicarbonate, 10 mM HEPES (4-(2-Hydroxyethyl, piperazine-1-ethanesulfonic acid), 33.3 mM D-glucose, 5.8 mM magnesium sulfate, 0.03 % BSA and penicillin/streptomycin (Gibco, Grand Island, NY). Explants were then cleaned to remove any remaining dura, and placed on a transwell membrane filled with media underneath. Median and ulnar nerves were obtained from wild type donor mice (C57Bl6) in the age range of p3 to p5 and abutted next to DRG explants to allow axons to traverse through for approximately one week to allow grafts to reinnervate.

4.4.2 Microfluidic Platform Preparation

A two-step photolithographic process was utilized to create the master mold. Silicon wafers (WRS Materials, San Jose, CA) were coated with SU-8 2002 (Microchem,

Newton, MA), spun, and soft baked using parameters specified by the manufacturer to yield a resist thickness of 2.5 μm. An array of microchannels were defined by UV light exposure through a high resolution transparency (Cad/Art, OR). The dimensions of each microchannel for standard devices were: width = 10 μm, length = 500 μm. The exposed substrate was once again baked, to enhance polymer cross-linking post exposure, and developed as stated in the resist technical sheet to fully define the microchannels. The process was immediately repeated with SU-8 3050 (Microchem, Newton, MA) to define the fluidic reservoirs with dimensions: width = 3 mm, length = 13 mm. The master mold was then treated with trichlorosilane (United Chemical Technologies, Lewistown, PA) for 30 minutes to create a nonstick surface for subsequent processing. Standard soft lithography was performed using Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning, Midland, MI) as described previously (Ng, et al., 2003). After curing, the PDMS was carefully removed from the master and access ports were created using dermal biopsy punch tools (8 mm) (Huot Instruments, Menomonee Falls, WI).

4.4.3 Collagen gel preparation

Collagen gels were prepared using a PDMS mold of roughly 50-100 microns in thickness. The collagen gel solution was prepared by mixing Collagen I, Rat tail (Gibco, Grand Island, NY), 7.5% sodium bicarbonate, and 10X PBS in a 8:1:1 ratio. If laminin was used, 10 ug/ml was added to the solution. All solution preparation

was carried out on ice and under sterile conditions. Once the mixture was vortexed, a 20 minute wait period was observed before loading the solution into PDMS molds in order to limit bubble formation. Gels were placed in a 37° C incubator for 2 hours to allow adequate time for gelation of the thin films. Gels were then cut to dimension as needed from the formed sheets and placed under transected nerves.

4.4.4 DRG Cell Experiments

DRG neurons were loaded into the soma side of devices and grown for sufficient time to allow axons to grow through channels and into the axonal compartment. Phase contrast imaging was used for a majority of experiments. Representative axon images and a certain subset of experiments were performed by labeling axons with viral GFP for enhanced visibility. Axons were transected at the base of the microchannels by gently scratching the surface of the glass with either a glass pipette or syringe tip, and neurotrophic factors were applied to axonal or neuronal cell body compartments at a concentration of 100 ng/ml, a commonly used peak concentration for various neurotrophic factors. For GDNF and cytochalasin D experiments, 100 ng/ml of GDNF was added to the axonal compartment concurrently with 10 nM of cytochalasin D. Regeneration of axons was monitored by daily imaging of the axonal side and measuring the length of longest axon coming from each microchannel. A minimum of 10 axons per experimental condition was measured and experiments were done in triplicates for GDNF family ligands. A minimum of 5 axons was done for

GDNF and cytochalasin D.

4.4.5 DRG Explant Experiments

After approximately one week in culture, allowing for enough time for nerve grafts to fully reinnervate and with a minimum of 2 days without GDNF, grafts were transected in order to mimic a nerve injury. Collagen gels with or without laminin were placed under transected grafts in order to overcome the intrinsic directionality of transwell membrane. Note that when with or without the presence of laminin is noted in this chapter, this refers to the presence or absence of laminin within the context of the collagen gel. GDNF was then either added or withheld after transection and during media exchange every two days, depending on the experimental condition. Explants were imaged under a fluorescent scope daily for 5 days. These images were stitched and quantified using a superimposable radial grid. This grid is adjustable and starts at a radius of 250 μm, continuing in 250 μm increments. In this way, axons that cross over at different lengths can be binned.

4.5 Results

4.5.1 Axotomy and Axonal Regeneration by Neurotrophic Factors

In order to identify the regrowth of axotomized axons, we allowed neuronal processes to grow into the axonal chamber and then transected axons using either a glass pipette or metal syringe. In Figure 4.5, we see a representative device, seeded at lower density in order for enhanced visualization of single axons, before and after axotomy. These neurons were transfected with viral green fluorescent protein (GFP) in order to enhance visualization. Note that while axons are cut slightly farther from opening, there is some retraction and degeneration. For our experiments, we added recombinant GDNF, neublastin, or neurturin to either axon or cell body compartments of cultured DRG neurons for 72 hours. Multiple images of the axons were captured using phase contrast microscopy and ImageJ (NIH; Bethesda, MD) was used to calculate percent changes in axon lengths before and after axotomy in the application of growth factors. In Figure 4.6, we see representative images of the DRG axons before and after different neurotrophic factor treatments, all taken at the same magnification. In these images, we see the axons exiting channels and traversing into the axonal compartment on the right.

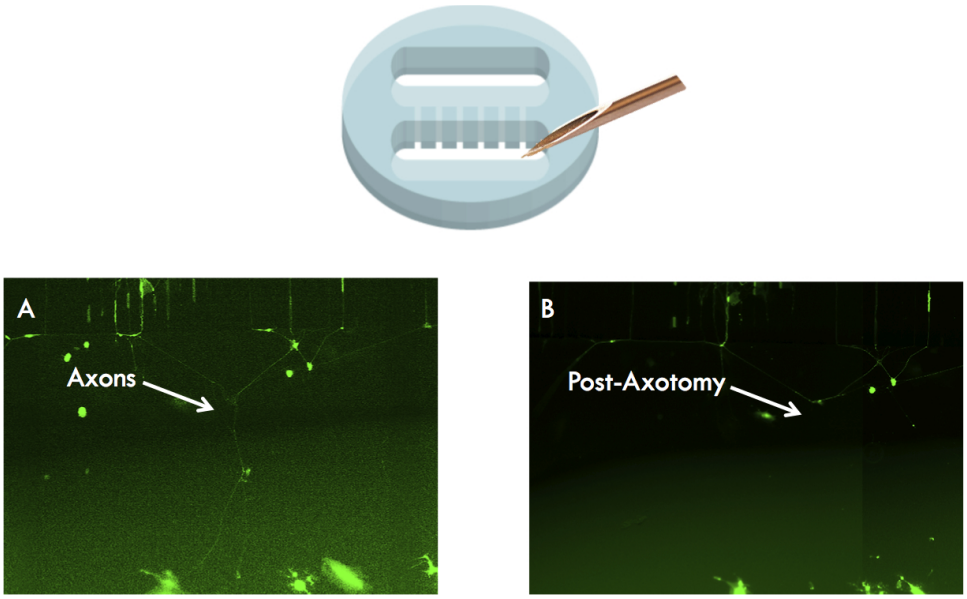

Figure 4.5: A schematic of axotomy performed with the device is presented above. Below this are representative images of axons A) before axotomy and B) after axotomy.

As summarized in Figure 4.7, all of the neurotrophic factors enhanced axonal regeneration whether they were applied to the axonal or somal compartments but GDNF was most potent. Furthermore, there was a slight benefit to applying GDNF to the somal compartment. In order to verify if the enhancement effect of adding GDNF to the axonal compartment was due to local mechanisms, we carried out experiments with concurrent application of GDNF and cytochalasin D. In Figure 4.8, we can see that the results indicate ($p < 0.05$) that this enhancement is diminished with the application of the retrograde transport blocker, indicating a need for GDNF to be transported into the cell.

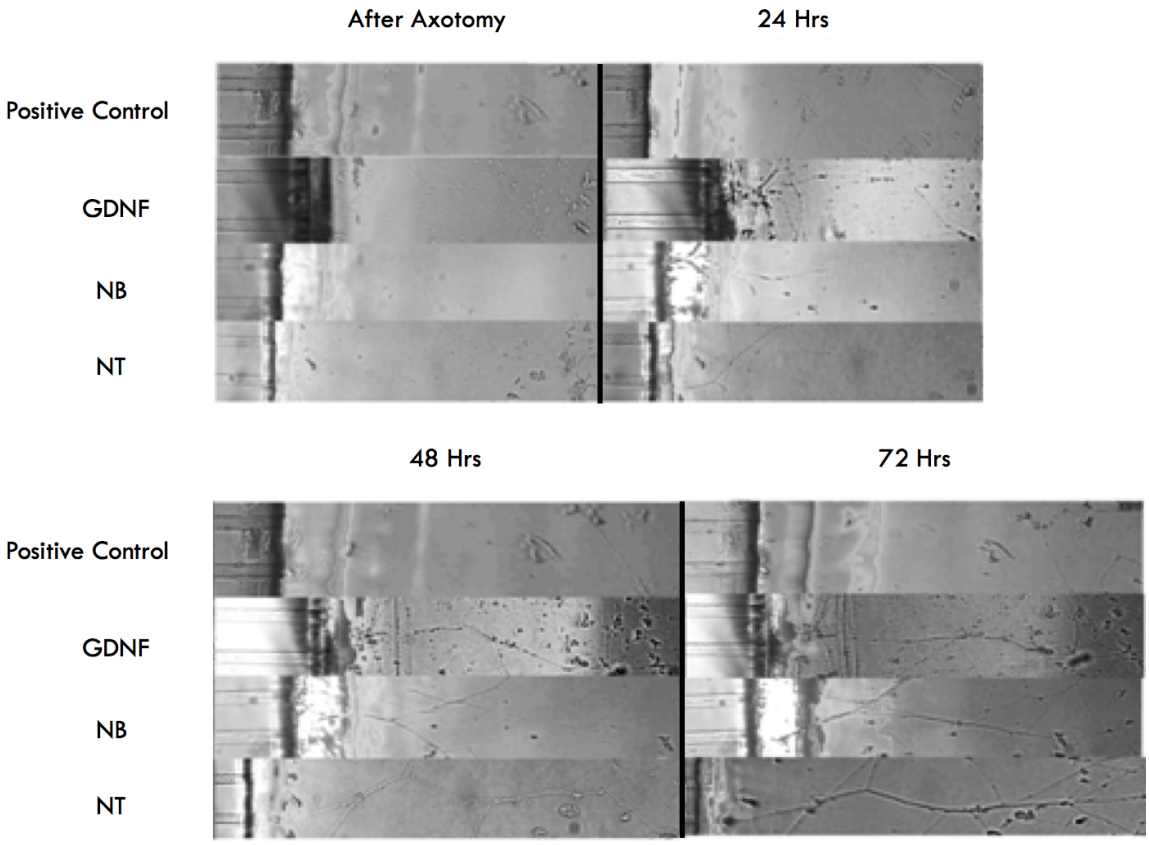

Figure 4.6: Representative phase contrast images of regenerating axons where neurotrophic factors were administered into cell body compartment. Images were taken every 24 hours for 3 days after injury. Immediately after axotomy no difference can be seen between the conditions. Over the next 72 hours, there is very little growth in the positive control compared to the growth factor treated conditions.

4.5.2 GDNF Effects at the Tissue Level

Explant cultures are more reflective of the *in vivo* environment than dissociated cultures as the DRGs can be cultured with support cells such as Schwann cells and macrophages. In order to determine if the enhancement of growth by GDNF translates to the tissue level, we cultured DRG explants with or without GDNF, and

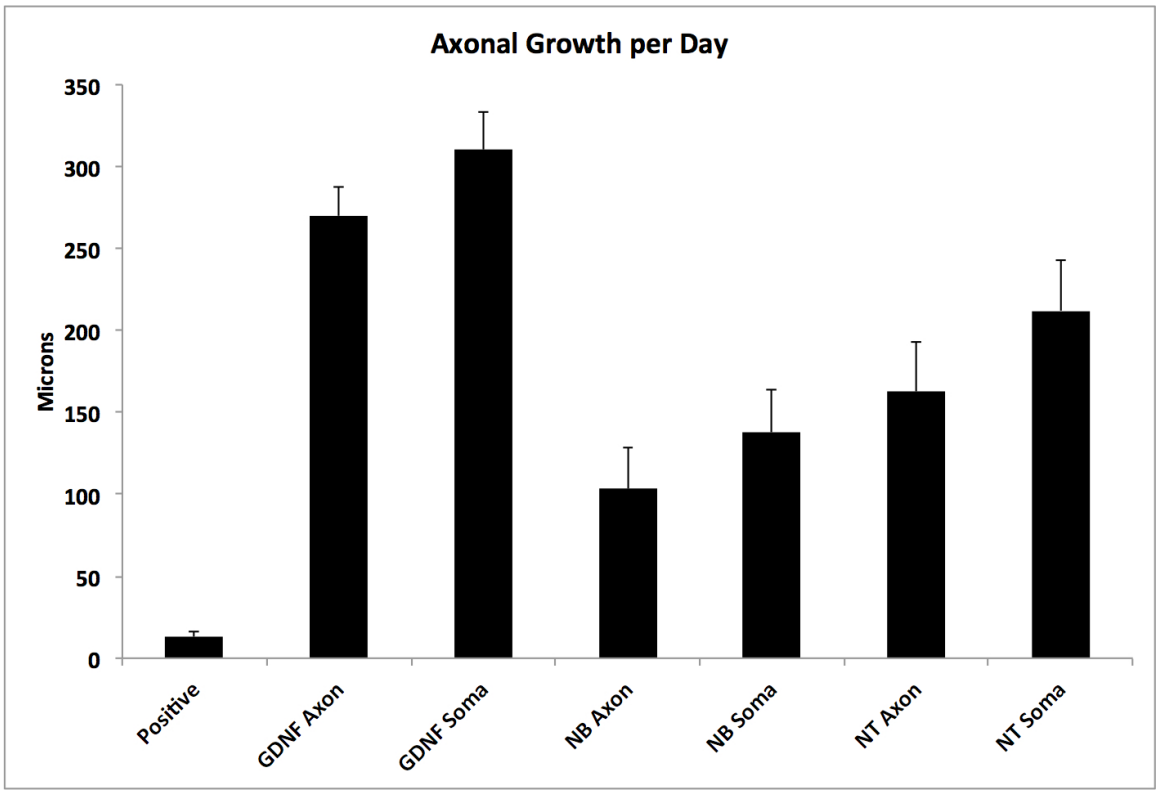

Figure 4.7: Axonal regeneration by GDNF, neurturin (NT) and neublastin (NB) after the axotomy. Rate of axonal regeneration induced by the neurotrophic factors over 3 days. All tested growth factors enhance axonal outgrowth compared to the positive control, applied to either location

with or without the presence of laminin. A representative culture immediately after transection and 5 days later with a radial grid super imposed to demonstrate our quantification technique can be seen in Figure 4.9. In Figure 4.10, we see axon counts at the different radii compared. These graphs allow us to observe that there are more axons that are at least 250 μm for both GDNF conditions and this effect diminishes over time. As we go to the larger axon lengths, particularly for axons at least 2000 μm, there are more axons for the GDNF without laminin condition, potentially due to the initial boost. Viewed another way in Figure 4.11, we can see growth over several

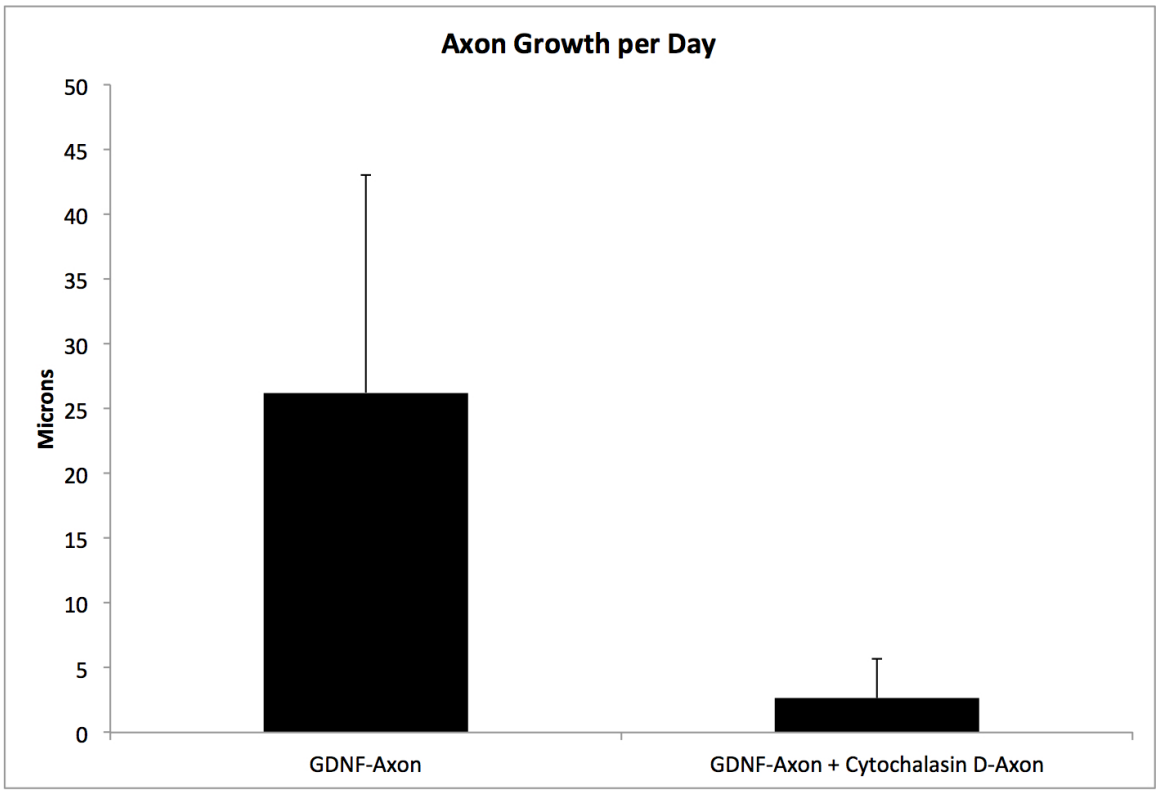

Figure 4.8: Axon regeneration with Axonal GDNF application compared with Axonal GDNF application concurrently with cytochalasin D

days compared between our four conditions of GDNF and laminin, GDNF without laminin, control with laminin and control minus laminin. An upward slope within our growth curve implies increased branching since this indicates there are more axons present at the next larger radii.

GDNF without laminin proved to have the largest amount of growth on the first day, particularly as compared to both controls ($p < 0.05$). We again see that this effect is not as strong over the remaining days, until day 5, where there is again a significant ($p < 0.05$) difference between GDNF without laminin and control with laminin.

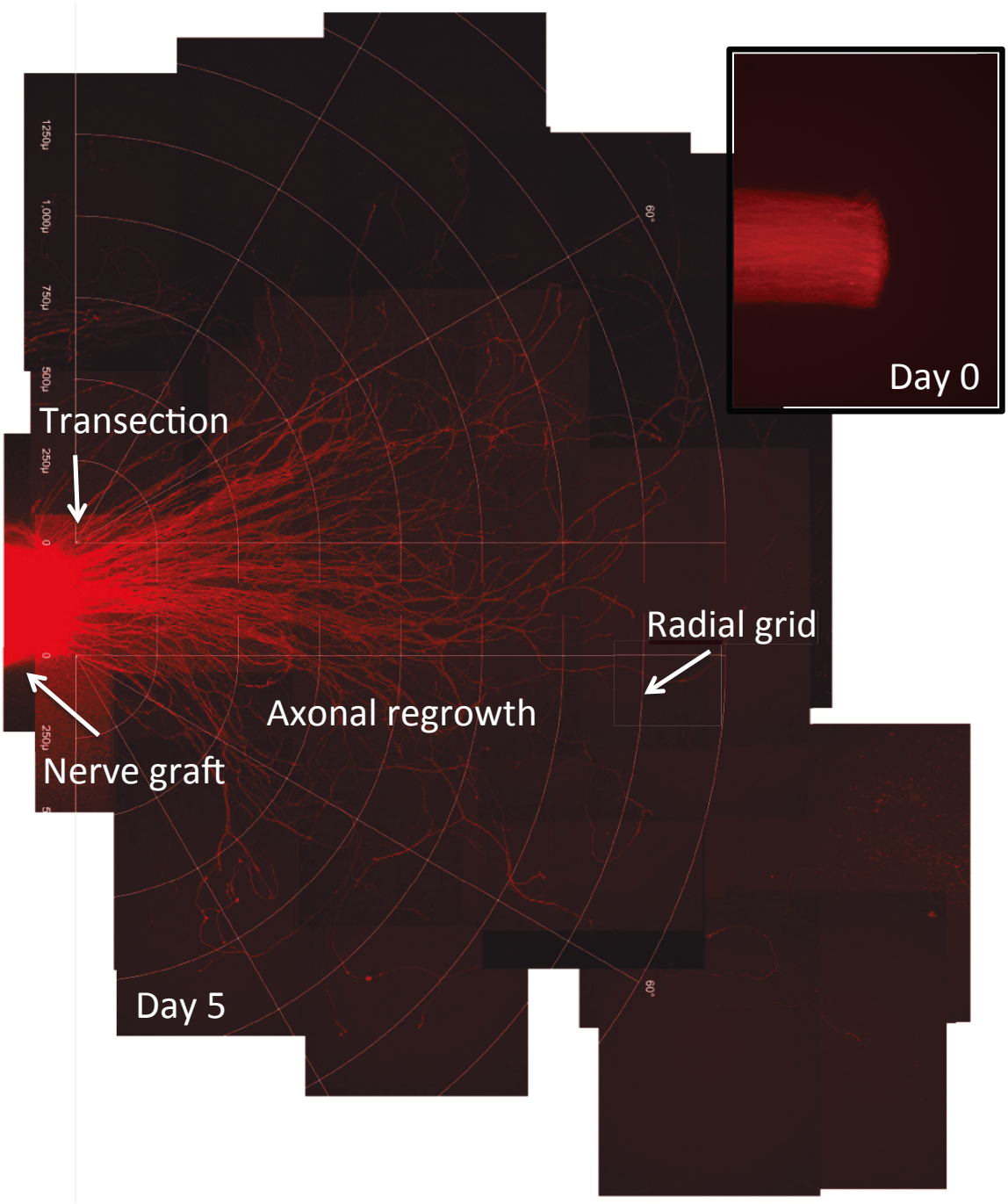

Figure 4.9: An example of a DRG culture immediately after nerve transection (inset showing Day 0) and the same culture 5 days after transection with a template superimposed to demonstrate how axonal growth was quantified. The grid is adjusted around the nerve graft, starting at the site of transection. The template starts at an inner radius of 250 μm from the site of injury and increases in 250 μm increments. All axons to the right of the point of transection are axons regrowing post-injury.

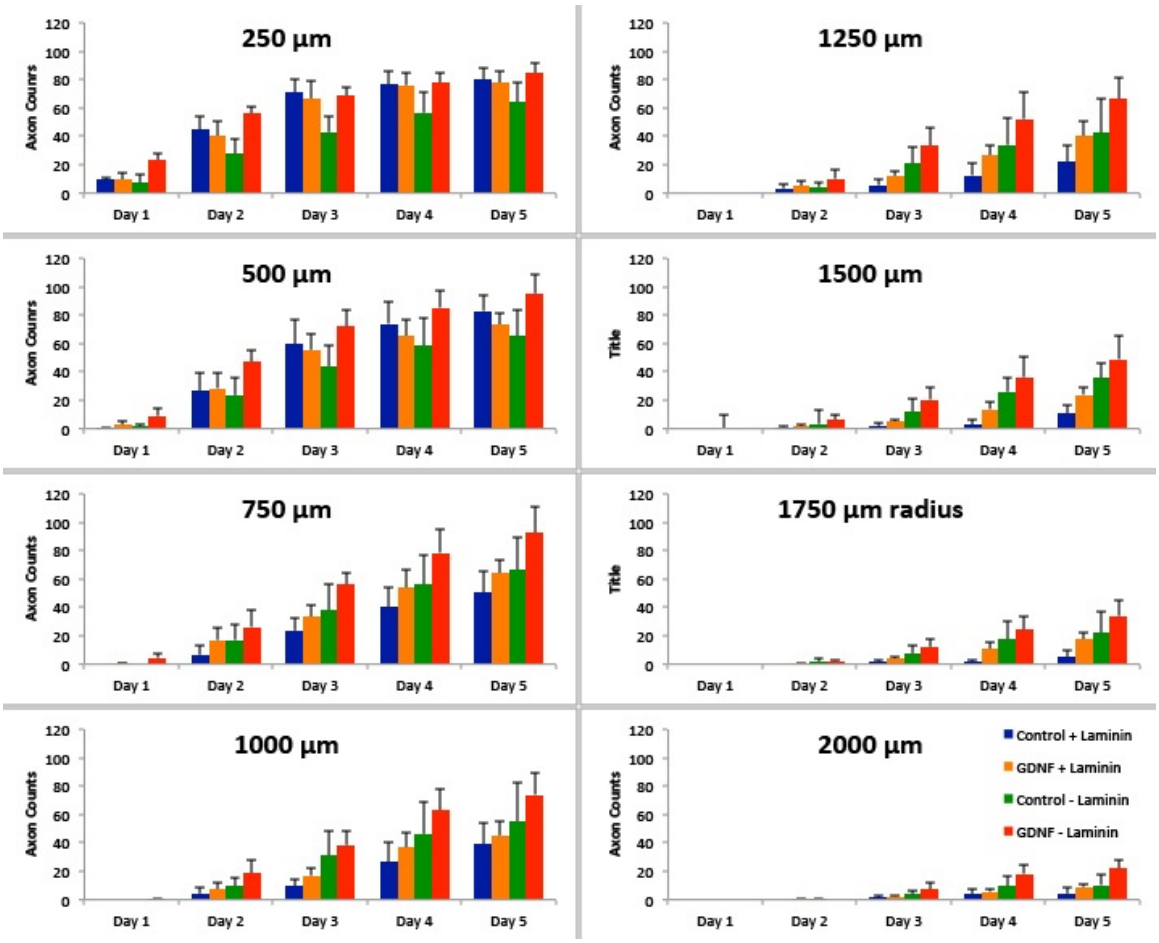

Figure 4.10: Quantification of axon counts separated by axon lengths, over 5 days in each graph. There are significantly more axons that are at least 250 μm by Day 1 and at least 2000 μm by Day 5 for the GDNF without laminin condition particularly as compared to either control condition.

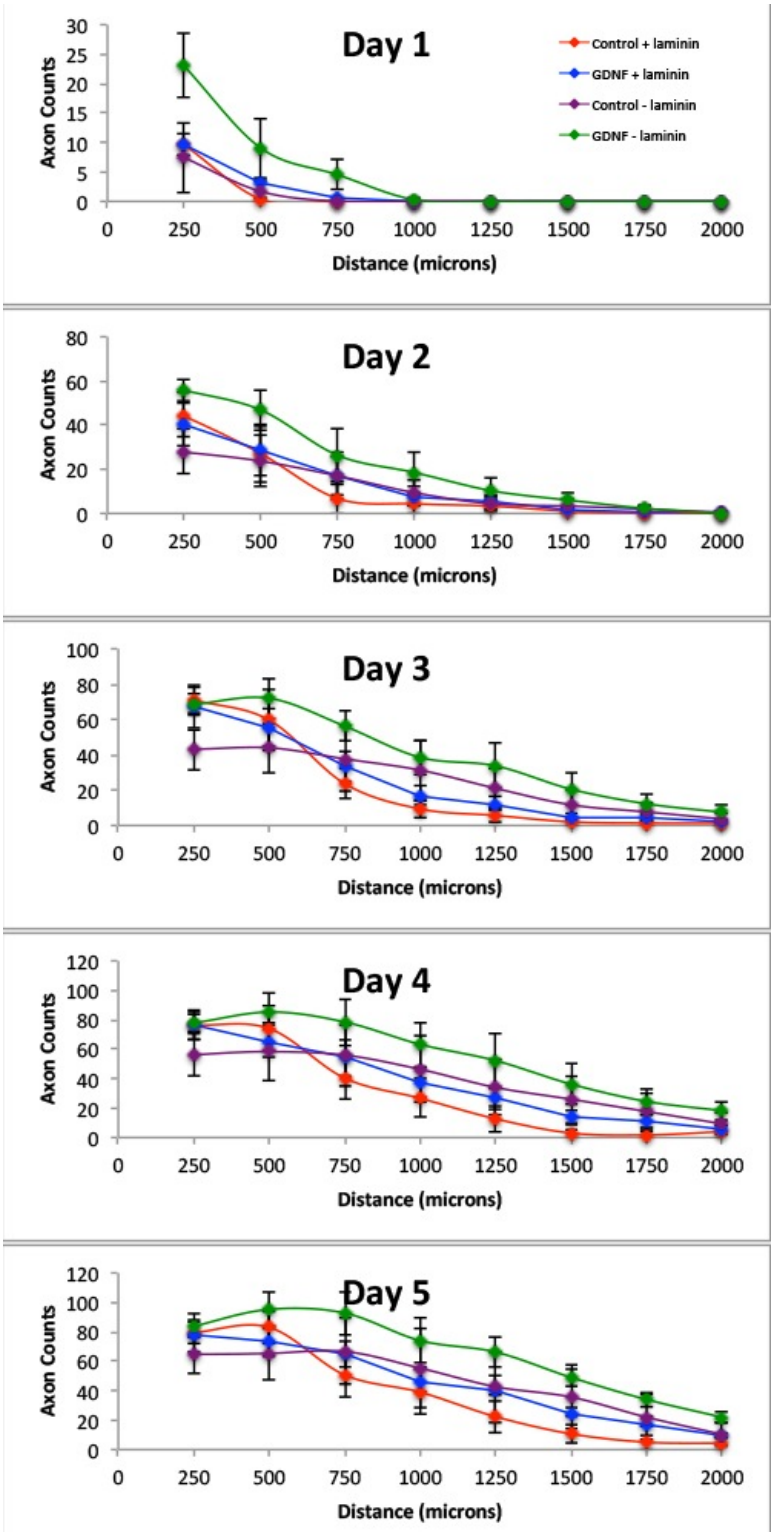

Figure 4.11: Quantification of axon counts at different lengths separated by day, allowing for visualization of growth curves, with the most pronounced difference on Day 1 for GDNF without laminin

4.6 Discussion

Compartmentalized microfluidic culture systems have been utilized in a variety of neuronal studies, from examining the effects of toxins and neuroprotectants on axons versus soma, to enhancing spatial and temporal control of neurons and other cultures, and performing axon-glia co-culture studies [6–8,82]. Several injury systems have been incorporated within microfluidic culture devices in order to investigate axon-specific mechanisms in injury and regeneration. These systems include simple aspiration of the distal compartment, two-photon laser ablation, and hydrodynamic shear based axotomy [5,79]. In the current study, through modification of the device into an open system, we were able to transect axons simply and easily by scratching the surface of the glass with a sharp pippette or syringe.

As compartmentalized microfluidic culture devices have become ubiquitous, appropriate characterization of the diffusion properties within the devices would not only be beneficial but necessary. This study demonstrates a potential pitfall in designing and carrying out microfluidic experiments with neuronal cultures. Unless a proper hydrostatic pressure is maintained, there is no true fluidic separation of the axonal and neuronal cell body compartments. Experiments studying the effects of individual local manipulations of axons and neuronal cell bodies will have to take these observations into consideration. This is especially true for small molecular manipulations as they are more likely to diffuse through the microchannels and confound the findings of an experiment.

Neurotrophins have traditionally been known to play integral roles in neuronal survival during development, but only relatively recently has their function in regeneration been explored [53]. GDNF and its family of growth factors, neurturin, artemin, and persephin, represent a class of novel neurotrophic factors. These growth factors signal through a two-receptor complex consisting of rearranged during transfection (RET) Trk and a glycosylphosphatidylinositol (GPI)-linked GFR-α. The growth factors GDNF, neurturin, artemin, and persephin preferentially bind to bind to GFR-α1, GFR-α2, GFR-α3, and GFR-α4, respectively. GDNF has been shown to provide neuroprotection and promote axonal regeneration, but the role of the other family members is not as clear.

Injury to peripheral nerve reactivates its intrinsic growth capacity, and the retrograde transport of injury signals has been suggested to be one of the essential mechanisms for regeneration [46]. The retrograde transport of GDNF has been postulated to act as a positive injury signal for induction of regeneration [176]. The enhancement of regeneration by GDNF within our in vitro system is consistent with previous studies. It has previously been demonstrated that GDNF selectively promotes regeneration of injury primed sensory neurons, both *in vitro* where GDNF caused enhancement of neurite outgrowth in preconditioned DRG neurons, and *in vivo* where GDNF administered directly to cell bodies in lesioned spinal cord facilitated the preconditioning effect and enhanced regeneration further [177].

In our study we found that GDNF acts as a more potent inducer of regenera-

tion than the other GDNF family growth factors we examined, and this finding is consistent with previous studies. GDNF and its receptor GFR-1 are upregulated in the distal denervated segment of injured nerve, suggesting that GDNF may provide trophic support for injured peripheral neurons. No analogous upregulation of neurturin, persephin, and artemin or their receptors was found following injury [178]. However, it is note-worthy that we do see enhancement of regeneration at all if this is the case.

The finding that GDNF administered to cell bodies produced better results than GDNF administered to distal axonal compartments is interesting. A previous study utilized compartmentalized cell culture devices to study the role of GDNF as a retrograde survival factor and its ability to promote survival over long distances to cell bodies [179]. In this study, it was found that GDNF promoted survival of DRG neurons equally well from either distal axon or cell body application. However, this study was not done within an injury model, and the DRG neurons were relatively healthy, and thus the reason for this discrepancy in the potency of GDNF depending on location of application may be due to the fact that we are using injured DRG neurons. It is important to note that the mechanism of action of GDNF may be different in these two systems, indicating the need to study the role of growth factors in both injury and developmental systems separately. It has been demonstrated that GDNF and GFR-1 are retrogradely transported in peripheral axons, but these studies were also done in relatively healthy neurons [180]. Axonal injury may have

an impact on protein turnover and retrograde transport, and this may impair some of the retrograde pathways for GDNF transport, making the application of GDNF directly to the cell bodies more effective. Our experiments comparing axonal GDNF application alone and together with cytochalasin D demonstrate the important role of retrograde transport of GDNF, as well as indicate that the enhanced regeneration effect may be cell body specific rather than at the localized axon.

Our experiments at the tissue-level were done with GDNF, which elicited the strongest response in our cellular studies. The results demonstrate that the regeneration enhancement effect of GDNF translates to an extent at the tissue level, but not nearly as dramatically, as the control explant cultures regrow much better than the control cell cultures. The fact that the enhancement effect seems to be most pronounced on Day 1 immediately following injury and application on non-laminin coated substrates can have implications for timing of future therapy applications of growth factors. It is important to note that after axotomy, there will be an upregulation of endogenous growth factors after injury that is more pronounced in a tissue level model as compared to cellular, and this may be why the effect of exogenous GDNF is either diminished or hidden. As GDNF is necessary for development, future studies involving DRG explants from conditional knockout mice may elucidate GDNF's role further. The demonstration of a slight increase in branching is consistent with other studies in which GDNF increases branching in different types of neurons to varying extents [181, 182].

While laminin is widely known to promote axonal outgrowth, it is interesting to see that the effect of GDNF when there is no laminin in the collagen gel is significantly higher than not only the control without laminin but also the control with laminin, as this indicates that laminin alone may not be sufficient to achieve optimal growth. This finding may also have implications for future treatments for nerve injuries in which the basal lamina is disrupted. While some studies indicate the necessity of laminin in order to promote axonal outgrowth in the presence of growth factors in *in vitro* studies, our results indicate that laminin may be a confounding factor [51].

It is also important to note that other significant data may not be as readily apparent due to large error numbers associated with these DRG explant cultures. This may be due to inherent differences in not only absolute numbers of DRGs but differences in the distribution of subpopulations of DRGs that are responsive to GDNF. A comparison between DRGs of the same spinal level may level the playing field to produce lower error. Preliminary experiments were performed noting spinal level of origin of the DRG explants, in our case L4-L6 (Lumbar level 4 to Lumbar level 6), and without the presence of laminin. The results from these experiments indicated that spinal level may be important in determining responsiveness to GDNF as well as intrinsic axonal outgrowth, however, a larger scale study is needed. This is an important area for future study, not only as it seems to be most appropriate to compare DRGs at the same level, but also because a relationship between spinal level and responsiveness to different growth factor treatments can affect an eventual out-

look for functional recovery based on corresponding level of injury. These findings can lead to tailorable treatments for particular nerve injuries based on spinal level origin of the nerve. In addition, performing these experiments within a tissue-level compartmentalized platform will help tease out if there are differential effects based on exposure to either solely the distal axon or cell body at the different spinal levels.

Neurotrophic factors are a promising area of research for understanding regeneration. Their role in providing trophic support during development and in maintenance of neurons has long been known, but elucidating their roles in regeneration may prove fruitful in the development of therapies for overcoming neural degeneration and for enhancing regeneration post-injury. Understanding axon specific or cell body specific effects of growth factors and being able to distinguish between local effects and retrograde signaling will be necessary for any future therapies. Compartmentalized microfluidic culture devices may be instrumental in these studies, but caution must be exercised to better characterize the devices to ensure true microfluidic separation of chambers. Progression of cellular studies to the tissue level bring us one step closer to actualizing clinical therapies for traumatic peripheral nerve injuries.

Chapter 5

A Two-Compartment Organotypic Model of Mammalian Peripheral Nerve Repair

This chapter has been published in the Journal of Neuroscience Methods [183] and reprinted according to the guidelines of the journal.

5.1 Abstract

Schwann cells in the distal stump of transected nerve upregulate multiple growth factors that support regeneration on a modality-specific basis. It is unclear, however, which of these preferentially support motor axons. Identification of these factors

will require a model that can isolate growth factor effects to growing axons while reproducing the complex three-dimensional structure of peripheral nerve. A two-compartment PDMS base is topped by a collagen-coated membrane that supports a spinal cord cross-section above one compartment. Fluorescent motoneurons in this section reinnervate a segment of peripheral nerve that directs axons through a watertight barrier to the second compartment, where nerve repair is performed. Motoneurons remain healthy for several weeks. The axons they project through the water-tight barrier survive transection and robustly cross a nerve repair to reinnervate an additional nerve segment. Fluidic isolation of the two compartments was confirmed with a dye leakage test, and the physiologic integrity of the system was tested by retrograde labeling of only those motor neurons to which tracer was exposed, and by limitation of toxin effects to a single compartment. In traditional monolayer cell culture, neuronal compartments cannot be isolated. Our previous in vitro organotypic nerve repair model accurately modeled nerve repair, but did not allow individual control of motoneuron and growth cone environments.This model isolates treatment effects to growing axons while reproducing the complex three-dimensional structure of peripheral nerve. Additionally, it facilitates surgical manipulation of tissues and high-resolution imaging.

5.2 Introduction

The role of pathway-derived growth factors in promoting motoneuron regeneration is poorly understood. Several growth factors are upregulated by denervated Schwann cells in the distal nerve stump soon after injury [54]. Recently, anatomical and functional subsets of these Schwann cells have been characterized by unique growth factor profiles that have been found to support regeneration of sensory and motor axons on a modality-specific basis [15]. Although upregulation of several growth factors differs between sensory and motor nerve, it is not clear which of these factors could be responsible for the modality-specific support of motor axon regeneration. Identification of these factors will require a model that can both localize growth factor effects to growing axons and duplicate the complex three-dimensional architecture of peripheral nerve.

Growth factor effects on regeneration are isolated most easily *in vitro*. Cell culture devices such as the Campenot chamber and its microfluidic counterparts are able to isolate growth factor effects to the growing axon. However, the three-dimensional configuration of extracellular matrix components is especially difficult to model *in vitro* [51]. As a result, currently available techniques cannot reproduce the three dimensional structure of nerve, and thus cannot model nerve repair accurately [4, 6].

Attempts to determine the role of pathway-derived growth factors in vivo are hampered by the complexity of the peri-axonal environment and by the paucity of relevant conditional knockout mice. Growth factors are produced not only by Schwann cells,

but also by infiltrating macrophages, central glia, neurons that synapse on the regenerating motoneuron, and by the neuron itself. These growth factors can also have multiple effects that influence regeneration indirectly, such as promoting neuronal survival, signaling axonal injury to the neuron, and modulating Schwann cell behavior during Wallerian degeneration [184]. Clearly, there is a need for a platform that selectively controls the growth factor environment within the three-dimensional structure of peripheral nerve. To address this need, our lab developed the first *in vitro* model of adult mammalian nerve repair in an organotypic co-culture system [10]. Organotypic cultures are prepared from nervous tissue without dissociation, and thus preserve the three-dimensional cytoarchitecture within both spinal cord and peripheral nerve [11, 185]. Additionally, organotypic culture of motoneurons overcomes the difficulties encountered when maintaining these cells in a monolayer environment [9].

In our previously described *in vitro* model of nerve repair spinal cord sections from mice expressing yellow fluorescent protein (YFP) in their motoneurons were co-cultured with freshly-harvested segments of peripheral nerve [10]. To reconstruct ventral roots, these nerve segments were opposed to the ventral portion of the spinal cord section adjacent to the motor neuron pool to promote the ingrowth of YFP-expressing motor axons. After a week in culture, once the new ventral roots had been reinnervated, they were transected and nerve repair was performed by opposing their cut ends to freshly-harvested nerve grafts. As initially described, organotypic cultures were grown on a Transwell© collagen-coated insert within a 6-well plate. The

height of the Transwell enclosure compromised our ability to perform microsurgery on the cultured tissue and to achieve the working distances required for high resolution imaging. The Transwell construct is designed to be imaged from below; image quality is degraded by the fluid and plastic beneath the membrane, and magnification is limited by the distance between lens and fluorescent tissue. Additionally, this construct did not permit selective manipulation of the nerve repair environment without simultaneously altering that of the parent neuron.

To overcome the physical limitations of the Transwell construct, the walls of the membrane insert were shortened to increase mechanical access to the membrane for microsurgery and imaging. Fluidic isolation of motoneuron and regeneration compartments was obtained by replacing the 6-well plate with a low-profile two-compartment poly(dimethylsiloxane) (PDMS) base. Motor axons were conveyed from the motoneuron compartment into the nerve repair compartment through reconstructed ventral root that passed through a water-tight barrier. The result of these modifications is a biocompatible organotypic system that facilitates tissue manipulation and photography while permitting individual control of motoneuron and nerve repair environments. Growth factor effects can be studied in each compartment by adding growth factors or by blocking growth factor function with antibodies or siRNA. This model also has the potential to facilitate studies of Wallerian degeneration, myelination, and axonal pathfinding.

5.3 Materials and Methods

5.3.1 Fabrication of culture system

The completed device consists of two principle components: a PDMS reservoir for culture medium that is divided into two compartments by a central partition, and a superimposed Transwell membrane (Corning, Acton, MA) that provides a surface for the growth of spinal cord and peripheral nerve co-cultures. The dimensions of these components are illustrated in Figure 5.1.

PDMS reservoirs are replicas from a master mold created by micromachining a negative relief of the device design into aluminum. Aluminum provides a robust surface for molding that is both reusable and economical. The overall process flow for creating the molds and assembling the device can be seen in Figure 5.2. The dimensions of the PDMS reservoir are determined by the configuration of the Transwell insert, the space required for media supply and exchange around the periphery of the insert, and the PDMS surface area required for adhesion to a glass substrate. The outer wall of the reservoir is circular with a 38 mm outer diameter and 33 mm inner diameter. The Transwell is suspended over the media by the central partition, and by two steps placed at 90 degrees to the axis of the partition. The partition is 28 mm long and 1 mm tall, and bisects the device into two separate compartments. In order to provide adequate support for the membrane and to ensure bonding to the glass substrate, the partition is 5 mm wide along most of its length. At the center of the

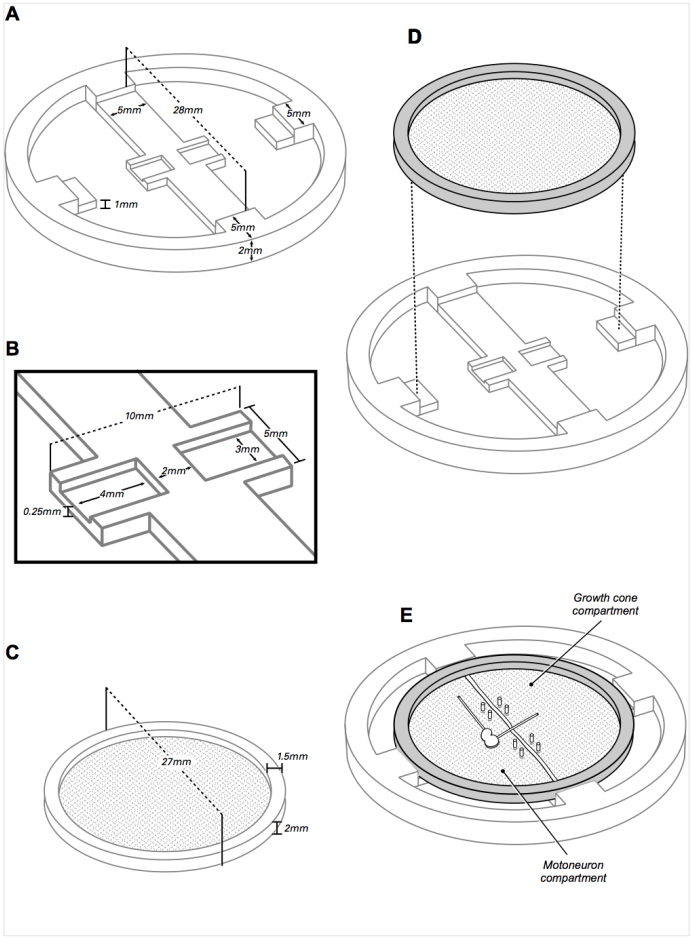

Figure 5.1: The two-compartment organotypic chamber consists of a PDMS reservoir (A,B) and a modified Transwell insert (C). The reservoir is bisected by a partition to create two fluidically isolated compartments. The Transwell insert is seated within the insert so that the membrane surface contacts the surface of the PDMS partition and two additional supports (D). To complete the fluidic isolation of the two compartments, the membrane is anchored to the partition with steel pins and bisected along the center axis of the partition (E). A thin bead of silicon grease is then placed to separate the cut edges of the membrane, insuring that no fluid can diffuse through the membrane from one compartment to the other. A spinal cord segment is placed in the motoneuron compartment; one reconstructed ventral root remains within that compartment, and the other is directed across the grease barrier into the regeneration compartment.

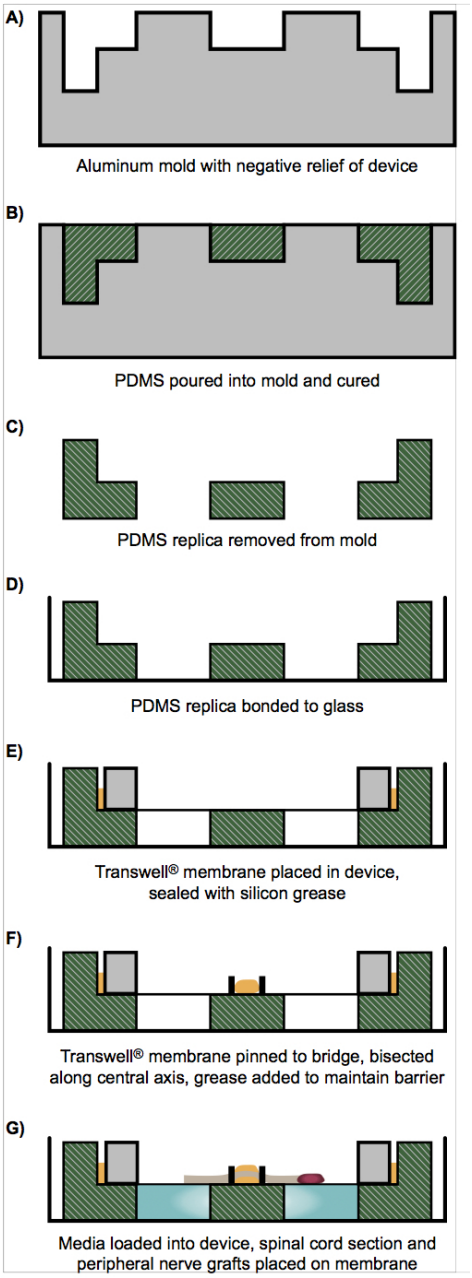

Figure 5.2: Device Process Flow. Device design is micromachined into aluminum and PDMS is used to create replicas. PDMS is poured into aluminum mold, cured, and removed. The created replicas are plasma bonded to glass substrates. A Transwell membrane is attached, bisected along its horizontal axis, and pinned to the device. A spinal cord slice is placed on one side of the device, with a control nerve in the same compartment and an experimental condition nerve traversing across the divider of the device to the other compartment.

device, a 3mm-long segment of the partition narrows to 2mm wide to maximize media circulation to the nerve as it passes through the grease barrier between compartments (Figure 5.1). To minimize sagging of the membrane beneath the spinal cord section, the central narrow segment of the partition is flanked by two 1mm-wide buttresses that extend perpendicular to its axis. A 0.25 mm thick film of PDMS connects the base of the buttresses to enhance their bonding to the glass beneath, and to promote the flow of media beneath the membrane so that bubbles do not form under the spinal cord explant.

The Transwell membrane receives additional support from two 5mm-wide peripheral steps that are aligned on an axis at 90°to that of the partition (Figure 5.1A). The lower stair step, which supports the Transwell insert, is 1mm above the floor of the reservoir, and thus in the same plain as the surface of the partition; the upper step is the full height of the device, and limits horizontal movement of the Transwell. The inner edge of the upper step extends 2.5 mm horizontally from the inner wall, creating a space 28 mm in diameter for the Transwell. Once a Transwell is placed into the device, the spaces around the Transwell between the steps and the partition serve as access points for media exchange.

Cross-linked PDMS (sold as Sylgard 184, Dow Corning, Midland, MI) is used to make replicas of the reservoir using standard soft lithography techniques as described in detail elsewhere (Xia and Whitesides, 1998). Figure 5.2 summarizes the device process flow. Briefly, the base and crosslinker are mixed thoroughly in a ratio of

10:1, and placed under vacuum to remove bubbles. The PDMS is then poured into the aluminum master mold, leveled, and cured (cross-linked) at 85°C for at least 1 hour. Once removed from the mold, these replicas are cured for an additional 72 hours to minimize uncrosslinked components and impurities that could be toxic to the spinal cord cultures. The PDMS replicas are then cleaned with ethanol, dried, plasma bonded to 50 mm-diameter glass bottom dishes, and retreated with plasma once bonded to ensure sterility.

The Transwell insert normally consists of a 24 mm diameter PTFE membrane with 3 μm size pores that is stretched over the open end of a 27mm-diameter polycarbonate tube. In our preparation the tube is transected 1mm from the membrane surface , leaving a 1mm thick rim of plastic that serves as a stretcher for the Transwell membrane (Figure 5.1C). This modified Transwell is placed atop the PDMS reservoir (Figure 1D), and anchored to the central PDMS partition with micropins fashioned from .2 mm wide steel wire (Fine Science Tools, Foster City, CA). Once anchored, the membrane is bisected with a no. 15 scalpel blade along the center axis of the partition to separate the two compartments, and a thin bead of silicon grease is interposed between the cut edges of the membrane to insure fluidic isolation (Figure 5.1E).

5.3.2 Preparing Organotypic co-cultures

Each assembled device was filled with enough serum-containing culture medium (50% minimal essential medium (Gibco), 25% HBSS (Gibco), 25% heat-inactivated horse serum (Hyclone, Logan UT), 25 mM HEPES, 35 mM D-glucose, 2 mM glutamine, penicillin/streptomycin (Gibco), 70 ng/mL of GDNF (R&D Systems Inc, Minneapolis, MN) to fill the bottom of the device and wet the membrane, approximately 500 μL per compartment.

Spinal cords were obtained from mice expressing a green variant of yellow fluorescent protein in sensory and motor neurons [186]. Transgenic B6.Cg-Tg(Thy1-YFP)16Jrs/J animals (Jackson Laboratories, Bar Harbor, ME) were maintained as heterozygous breeders (line thy1-YFP-H). Spinal cord sections were obtained from day 3 or day 4 postnatal mouse pups using a modified version of described methods [185]. Spinal cords were dissected from the pups, and their dura and root fragments removed. The lumbar spinal cord was cut into 350 μm-thick slices with a McIllwain tissue chopper (Ted Pella, Redding, CA). A single slice was placed in each device 2-3mm from the grease barrier with the motoneuron pools oriented closest to the barrier. The median and ulnar nerves of the sacrificed pup were harvested and their cut ends were each abutted to one of the two motor neuron pools in the spinal cord section to reconstruct ventral roots. One ventral root extended within the same compartment as the spinal cord parallel to the partition, while the other crossed the partition into the second compartment. Cultures were incubated at 37°C in a humid-

ified atmosphere containing 5% CO2. Medium was changed every other day, with the first two changes including 70 ng/ml of GDNF.

The compartment containing the spinal cord section is referred to as the motoneuron compartment, and the contralateral compartment is referred to as the growth cone or regeneration compartment. Applying a treatment to the motoneuron compartment will alter the environment of all motoneurons as well as the axons regenerating in that compartment, but will not change the environment in the regeneration compartment. Similarly, changing the environment in the regeneration compartment will not alter that of any motoneurons, or of the nerve regenerating in the motoneuron compartment.

Nerve repairs were performed five to seven days after the co-cultures had been established, by which time the ventral roots were re-populated with fluorescent axons. The reconstructed ventral roots were transected with microscissors 3-5 mm from the spinal cord. Median and ulnar nerves harvested from wildtype (C57BL) donor mice were then sharply transected and their cut surfaces opposed to those of the ventral roots. The axons in these grafts do not contain fluorescent protein, so all fluorescence in the grafts will represent regenerating axons.

5.3.3 Testing Fluidic Isolation

We sought to confirm the fluidic isolation of the two compartments. Trypan blue (Gibco, Grand Island, NY) was applied to the nerve repair compartment in

cultures that had already been maintained for 3 weeks, with regular media exchange every other day. The stock 0.4% by w/v Trypan blue was diluted to 0.01%. The motor neuron compartment continued to contain culture media. Cultures (n=3) were monitored over an additional two days.

5.3.4 Testing Physiological Integrity

Retrograde labelling was used to determine the relative ability of motoneurons to project axons through the grease barrier and into the regeneration compartment. The reinnervated ventral root in the regeneration compartment was transected sharply, and its cut end placed on a 1 mm square of stretched Parafilm. Crystals of Fluoro Ruby (Molecular Probes, Eugene, Oregon) were placed on the freshly-cut end of the graft for 30 minutes, after which the remaining tracer was carefully removed to prevent contamination of the medium.

We tested the physiologic integrity of the regeneration compartment by adding Nocodazole (Sigma Aldrich, St. Louis, MO) to inhibit microtubule polymerization. The Nocodazole was dissolved in dimethylsulfoxide (DMSO) (Quality Biological, Gaithersburg, MD) to a concentration of 33.3 mM, then further diluted in media to working concentrations. Based on previous studies, a 1μM concentration of Nocodazole was used initially. When this was found to have little differential effect, the nerves in both compartments were recut, and a 5 μM dose of Nocodazole was applied to the regeneration compartment. The media was replaced every other day in both

compartments for 1 week (n=3).

To evaluate the permeability of cultured nerve to large molecules such as antibodies and growth factors, we added FITC- dextran (150 kDa; Sigma-Aldrich, St. Louis, MO) to the media at a concentration of 1mg/ml for 24h. Nerve segments were then fixed for 2 h in 4% paraformaldehyde in Sorensen's buffer, stored overnight in 20% sucrose in Sorensen's buffer, and sectioned longitudinally at 10μM with a cryostat. Slides were then overlaid with coverslips using DPX (Sigma-Aldrich) to minimize fluorescent background.

5.3.5 Imaging

Real-time images were obtained with a Nikon Eclipse E600 fluorescent microscope equipped with a motorized stage, a spot CCD Camera, and an on-stage incubator that maintained required environmental culture conditions, including temperature, CO2 level, and humidity, to enable long-term monitoring and image acquisition. Using NIS-Elements Microscope Imaging Software, a stack of images was generated at progressive focal planes through the specimen. These stacked images were then flattened to a single image. Photoshop was used to adjust the contrast and brightness of individual flattened images and to create composites of several adjacent images.

5.4 Results

5.4.1 Viability of organotypic cultures in compartmentalized PDMS reservoir devices

Spinal cord and peripheral nerve are cultured on the upper surface of the membrane. They are nourished by underlying media that diffuses up through membrane pores, and have direct access to oxygen on their exposed upper surfaces. The media is replaced every two days through openings around the outer circumference of the device. Fluorescent images of a 15-day culture are shown in Figure 5.3. Motor neuron pools are densely populated with neurons that show no morphologic signs of degeneration (Figure 5.3B), indicating adequate nourishment and oxygen supply. Similarly, motor axons in both compartments appear healthy in that they are smooth, continuous fibers with no beading or interruptions that would indicate degeneration. The portion of the reconstructed ventral root that conveys axons into the regeneration compartment does not receive direct nourishment, as it is isolated from underlying media by the grease barrier. Axons in this area appear healthy none the less, suggesting that longitudinal diffusion of media within the nerve is sufficient to maintain viability over short distances.

Reconstructed ventral roots are populated with fluorescent axons 5-7 days after the cultures are set up, an interval similar to that observed in single-compartment cultures

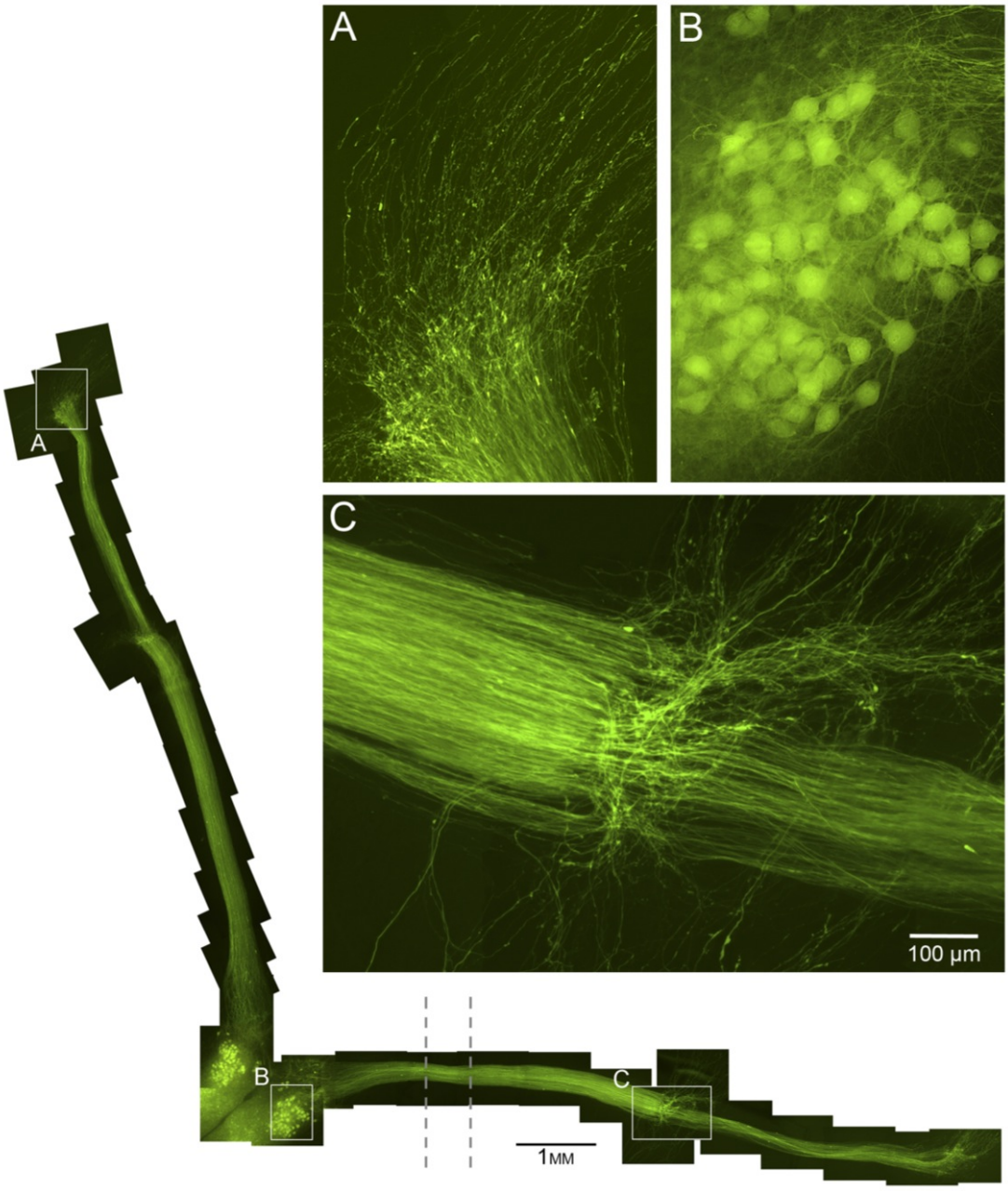

Figure 5.3: Reconstruction of an organotypic preparation during nerve graft reinnervation (left and bottom) from a representative 15-day culture. Dotted lines indicate location of the barrier. A. Axons that have traversed the vertical nerve graft growing out onto the membrane surface. B. Motoneuron pool that projects axons across the barrier into the regeneration compartment remains healthy. C. The ventral root-graft interface.

[10]. Once this has occurred, nerve repairs are performed in both compartments by transecting the ventral roots and opposing nerve grafts from wild type mice to their cut surfaces (Figure 5.3C). Reinnervation of the grafts was comparable in both compartments, as shown by the similar calibre of the reinnervated grafts (Figure 5.3). Grafts were reinnervated in 4-6 days, a slightly longer time than that the average 2-3 days observed in single-compartment cultures. Many axons cross to reinnervate the graft, but those not directly opposed to distal nerve grow out on the membrane in both compartments.

5.4.2 Fluidic Isolation

A critical attribute of the culture device is the ability to maintain fluidic isolation of the two compartments for the duration of an experiment. We reasoned that leakage was most likely to occur after the device had been handled several times for media exchange. We thus placed Trypan blue in the nerve repair compartment of devices that had been maintained for 3 weeks. The devices were observed closely for an additional 48 hours, and no leakage was observed (Figure 5.4). The device is symmetric along the bridge, with motoneuron and nerve repair compartments identical to each other, thus selection of a compartment for motoneuron or nerve repair is random. To confirm diffusion did not occur in either direction, we have also filled randomly selected compartments in multiple devices maintained for several weeks with dye and investigated for diffusion, as well as alternated which compartment dye was applied

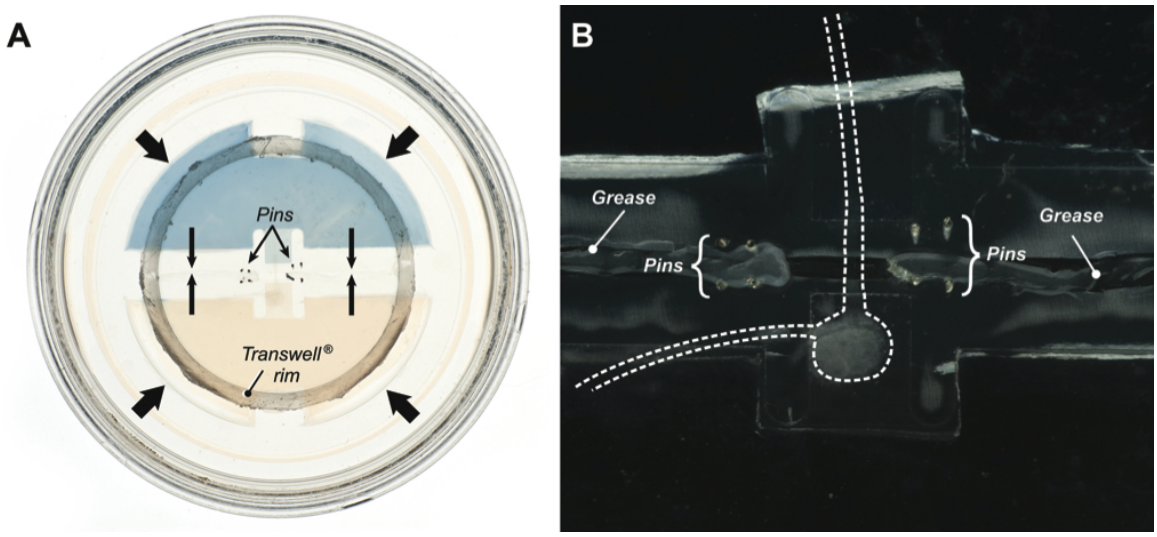

Figure 5.4: Fluidic Isolation in Device. The extent and duration of fluidic isolation were tested by placing a solution of Trypan blue in the regeneration compartment. A. View of an entire device, showing confinement of Trypan blue to the regeneration compartment after 3 weeks, a duration longer than most of our experiments. B. Darkfield view of the same device at higher magnification to illustrate the location of the spinal cord segment and ventral roots in relation to the grease barrier and steel pins.

in devices containing cultures. The results were identical to those pictured in Figure 5.4A.

5.4.3 Physiologic integrity of the dual-chamber system

FluoroRuby was applied to axons in the regeneration compartment to label their cell bodies in the motoneuron compartment. This tracer is taken up by cut axons, is retrogradely transported through the nerve and across the barrier, and accumulates

within parent motoneurons. During the process of regeneration, individual motoneurons may generate multiple collateral sprouts [187]. A small number of motoneurons could thus fully reinnervate the reconstructed ventral roots. In the dual-chamber system, all YFP-positive motoneurons in the pool that projected to the regeneration compartment were also labelled with Fluoro-Ruby, indicating that they had all projected axons across the barrier (Figure 5.5). Furthermore, retrograde labelling was seen only in the motoneuron pool, indicating that other neuron types had not projected axons into the graft.

To test the physiologic integrity of the two compartments, we blocked microtubule polymerization in the regeneration compartment with 5 μM Nocodazole. The representative images in Figure 5.6 were taken after one week of Nocodazole exposure. Ax-

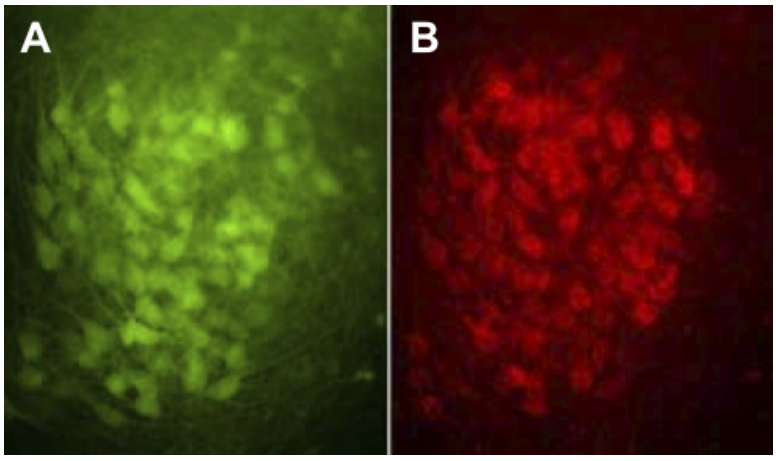

Figure 5.5: Fluorescent images of the motoneuron pool used to populate the regeneration compartment. All YFP-positive motoneurons in the pool (A) are also labeled with Fluoro-ruby (B). Axons in the regeneration compartment are thus linked to the entire motoneuron pool, rather than to a subset of neurons that might differ in some way from the others. Close inspection reveals that not all motoneurons in the pool expressed YFP, as occasional motoneurons are labeled with Fluoro-Ruby alone.

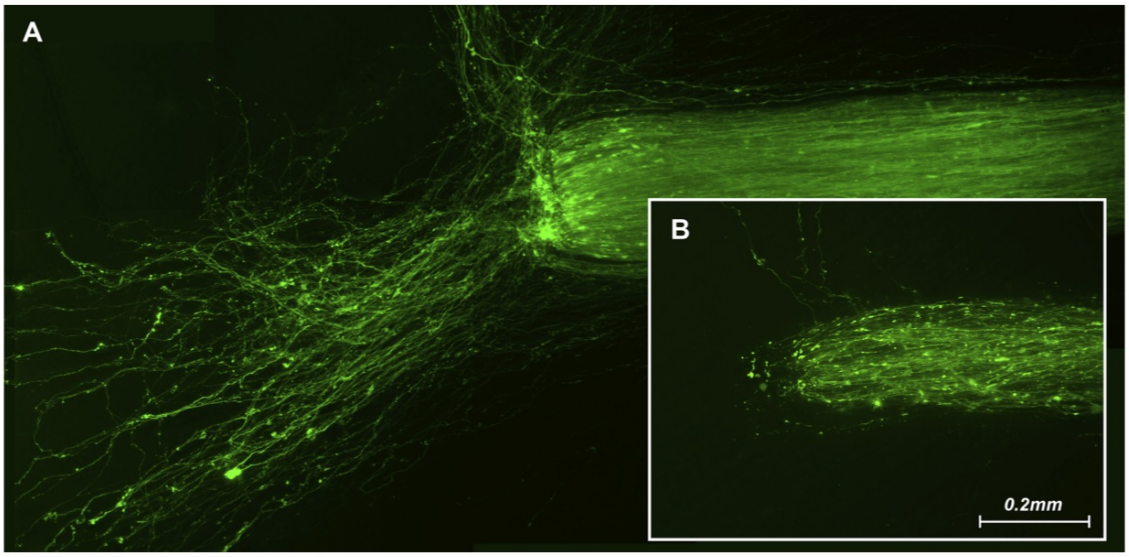

Figure 5.6: The ends of reconstructed ventral roots that had been transected 1 week previously. Nocodazole was added to the regeneration compartment at the time of nerve transection. In the untreated motoneuron compartment robust regeneration is evident, with many axons growing out on the membrane surface (A). In the treated regeneration compartment, however, axons are degenerating (B).

ons in the untreated motoneuron compartment regenerated vigorously (Figure 5.6A), while those in the treated regeneration compartment were degenerating (Figure 5.6B). Isolation of the treatment effect to the regeneration compartment was confirmed by the healthy appearance of both motoneuron pools (Figure 5.7).

Cultured peripheral nerve that had been exposed to 150kDa FITC-dextran at a concentration of 1 mg/ml for 24 h was uniformly fluorescent when viewed through a FITC filter, confirming that large molecules could access the endoneurial space.

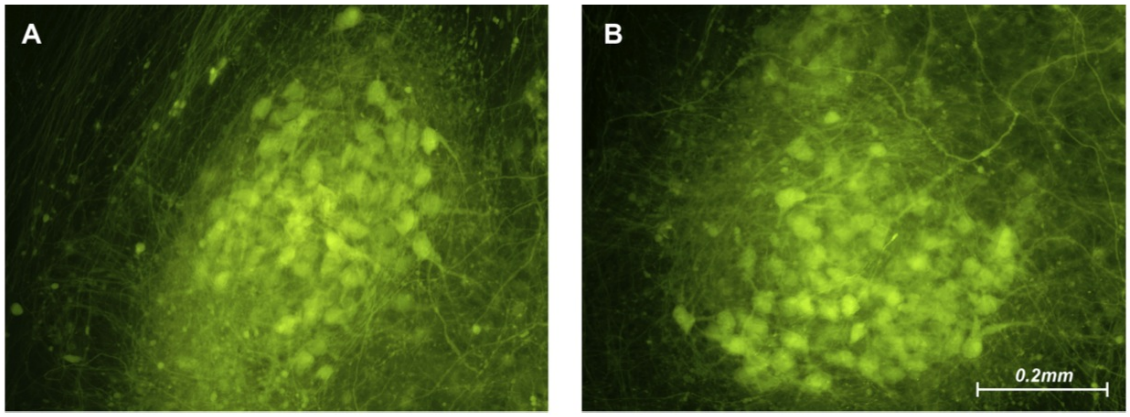

Figure 5.7: Motoneurons are abundant and healthy-appearing in both pools, indicating that Nocodazole has not leaked from the regeneration compartment into the motoneuron compartment.

5.5 Discussion

In our dual-chamber organotypic culture platform, YFP-expressing motoneurons in one chamber project their axons into a second chamber through a three-dimensional segment of peripheral nerve. Nerve repair is then performed in the second chamber by transecting the axon-bearing nerve and joining it to an additional nerve segment. In this system, the environment of either growth cone or motoneuron can be manipulated individually to localize signaling events that contribute to regeneration. The consequences of these manipulations can then be monitored by repeated fluorescence imaging of the YFP-positive axons as they regenerate.

Our construct is the three-dimensional descendant of the Campenot Chamber, a system for isolating neuronal cell bodies from their regenerating axons in monolayer cell culture [4]. In this on-slide device, axons elongate beneath a grease barrier that

separates neuronal and growth cone compartments by following grooves scratched onto the glass surface. The Campenot Chamber was used extensively in early studies of growth factor signaling. In cultures of NGF-sensitive sympathetic neurons, for instance, Campenot demonstrated that NGF promoted neurite outgrowth when applied to the growth cone, but not when applied to the neuronal cell body [188]. Adult DRG neurons could also be studied because of their relative ease of culture, but difficulties in culturing motoneurons precluded their use with this device [4, 189–191].

More recently, it has become possible to isolate neurons from their axonal growth cones by controlling the flow of medium rather than by imposing a physical barrier between them [5]. In these microfluidic devices, two fluid-filled chambers are connected by microchannels that direct axon growth. Because of the high resistance to fluid flow inherent in such minute channels, a small but sustained flow of medium is sufficient to counteract diffusion in the opposite direction, effectively preventing a substance added to the downstream chamber from entering the upstream chamber. These types of devices and their derivatives have been useful in a variety of neurobiology studies, including examination of local toxic effects, such as that of paclitaxel on sensory axons, as well as those of focal injury [7, 8, 24, 82, 86]. These devices also range from those that support multiple parallel experiments in a single device to those that allow for single axon injury [82, 100]. Although monolayer cell culture techniques that isolate neurons from their growth cones have been critical to our understanding of intracellular processes, they cannot model the complex three-dimensional inter-

actions necessary for peripheral nerve regeneration. Furthermore, these techniques are difficult to apply to motoneurons because of their need for trophic support from surrounding glia [190, 192].

Organotypic co-culture of spinal cord and peripheral nerve overcomes many of the shortcomings of monolayer techniques. Motoneurons that retain contact with glia and other motoneurons in spinal cord slices support robust regeneration and survive for up to three months [185]. Similarly, peripheral nerve survives in culture, and maintains the three-dimensional relationship of Schwann cells and basal lamina critical to the support of axon regeneration [193,194]. In our previous work, we co-cultured these two tissues and were able to innervate segments of peripheral nerve with motor axons [10]. The ability to contain these axons within the confines of a peripheral nerve segment, and thus independent of the membrane surface, suggested the possibility of using nerve as a flexible conduit to direct axons from one compartment to another.

Our initial model of organotypic nerve repair utilized the Transwell culture system [10]. In the current model, a shortened Transwell insert is supported by a two-chamber PDMS reservoir. These modifications enhance the quality of imaging, facilitate surgical manipulation of the tissues, and create separate motoneuron and regeneration compartments. In the Transwell system, cultures are imaged from below, through both the plastic bottom of the dish and 1mm of culture medium. Reducing the height of the insert facilitates high resolution imaging from above the membrane, eliminating the distortion caused by plastic and medium and allowing the microscope

objective to get closer to the tissue. Without the high walls surrounding the insert it is easier to manipulate tissues on the membrane with microsurgical instruments.

The greatest challenge in modifying our original model was to develop a partition that would simultaneously provide fluidic isolation of two compartments yet support the transfer of living axons from one chamber to the other. After trying several configurations, we succeeded by bisecting the membrane along the axis of the partition and separating the edges with a thin barrier of silicon grease. The consequences of modifying the membrane in this way included the need to pin the membrane to the partition and support it with lateral buttresses to keep it taut beneath the spinal cord and nerve segments.

As a result of these modifications, we have developed the first two-compartment organotypic spinal cord and peripheral nerve co-culture platform. We have demonstrated that motoneurons remain viable within this system, and extend their axons from one compartment to the other. Furthermore, we have confirmed the physiologic integrity of the system by applying nocadazole to inhibit axonal regrowth in one compartment without affecting axons or motoneuron cell bodies in the other compartment. With these advances, we can now isolate growth factor effects to regenerating motor axons without influencing their parent neurons. This can be done by adding the growth factor of choice to the regeneration compartment, or by perturbing growth factor function with antibodies or specific inhibitors. Additionally, work is ongoing in our laboratory to combine spinal cord and DRG cultures to produce mixed nerve

for studies of regeneration specificity.

5.6 Conclusions

We have developed a two-compartment culture device for the study of motor axon regeneration after nerve repair. The device consists of a two-compartment PDMS base to which a collagen-coated membrane is attached. We have demonstrated that such a device can be used to support viable spinal cord and peripheral nerve co-cultures, to perform nerve repair in vitro, and to maintain fluidic isolation between motoneuron and growth cone compartments. This device is ideal for the study of growth factor effects on axons regenerating within the three-dimensional structure of peripheral nerve.

Chapter 6

Discussion and Future Directions

6.1 Summary and Significance of Results

The overall objective of this dissertation was to develop and apply *in vitro* devices that enable precise control over the immediate environment of injured peripheral axons in order to determine localized effects of both degenerative and regenerative compounds. These devices enable a more complete understanding of peripheral axon injury and regeneration. This dissertation includes microfluidic devices utilized to determine the site of action of toxic, neuroprotective, and neurotrophic effects in peripheral axons, and culminates in the development of the first compartmentalized tissue-level nerve repair platform.

The study in Chapter 3 deals with chemical injury and aimed to examine the localized effects of the degenerative chemotherapeutic agent paclitaxel and determine

the site of action of the theorized neuroprotective glycoprotein hormone erythropoietin through the utilization of a compartmentalized microfluidic chamber. We demonstrate that paclitaxel is much more toxic when applied directly to the axon versus the cell body, causing degeneration within 24 hours. We also demonstrate that erythropoietin is capable of rescuing axons from this degenerative effect, interestingly through either cell body or axonal application. Importantly, this study demonstrates that axonal degeneration through local mechanisms can be counteracted through cellular mechanisms, which has implications for drug therapy development for polyneuropathies.

Chapter 4 first aims to more fully characterize the diffusion profile along microchannels of the chamber and establish sufficient height fluid height differences for sufficient hydrostatic pressure for use in longer term experiments. Through theoretical, computational, and experimental verification, a height difference of 2 mm was sufficient to localize compounds of relevant molecular weight to one side of the axonal chamber, and needed to be replenished every 24 hours to maintain isolation. Once this diffusion profile was established, the device was used as an injury device, where gently scratching the surface of the open axonal compartment was sufficient to cause a focal axonal injury and subsequent degeneration. The GDNF family of ligands was investigated as enhancers of regeneration post-injury due to their therapeutic potential. Interestingly, we find that all tested GDNF ligand family members have an effect on enhancing regeneration. As expected, GDNF was the most potent of these, with

enhanced effectiveness when applied to the cell body side. This study demonstrates the importance of understanding axon specific or cell body specific effects of growth factors necessary for future growth factor-based therapies for enhancing regeneration. Extension of these experiments to DRG explants demonstrates that the enhanced complexity at the tissue-level, including the presence of more support cells and ECM components, may affect or mask the regenerative effects of exogenous GDNF.

Finally, Chapter 5 deals with the development of a two-compartment tissue-level device that enables the control of the local environment of regenerating adult motor axons within a three-dimensional nerve repair model. It is the first device of its kind as a two-compartment spinal cord and peripheral nerve co-culture platform. Spinal cord is plated in the motor neuron compartment, while one nerve is placed in the same compartment to serve as a built-in control, and another travels across a barrier into the other chamber for examining localized effects. We have optimized the device design to ensure motor neuron viability while maintaining functional fluidic isolation between the compartments. We demonstrate successful axonal regrowth within peripheral nerve over the barrier, and robust and comparable regeneration across nerve repairs performed in both compartments. The physiological integrity of the device has been confirmed. First, this confirmation was done through dye isolation. Next, we added nocodazole, another chemotherapeutic agent known to cause axonal degeneration, and demonstrated that its effects could be localized to one compartment.

6.2 Future Directions

There is a need to study neurons in an environment that more closely resembles their intrinsic *in vivo* environment, where cell bodies and distal axonal ends may experience drastically different environments. This difference has implications for drug delivery for therapies aimed at polyneuropathies in terms of site of application, as well as for administration of growth factors in order to achieve prime regeneration enhancement. The studies performed in this thesis further demonstrate site specific effects and establishes a platform that more closely resembles a clinical nerve repair environment.

Developing a way to guide regenerating axons is a necessary next step in the future, as misdirection of regenerating axons remains a predominant obstacle to functional recovery post-injury. To enable incorporation into our two-compartment organotypic chamber, which has an intrinsic directionality due to the collagen-coated Teflon membrane, we have examined the use of gels on top of which to grow the axons, including collagen-laminin gels, as discussed in Chapter 4. Currently available technologies also enable the use of gels with pre-established growth factor gradients, and these can be used to examine the difference between soluble and bound cues. Current studies are underway combining spinal cord slice and DRG ganglion cultures so that both extend their axons into one femoral nerve graft. As an extension of guidance studies, developing a system to separate sensory versus motor axons with specificIty is another goal. Motor neurons and sensory neurons may also exert an effect on each other in

terms of both tropism and trophism and this is something that can be investigated within our device.

There are several additional exciting areas for future studies. These areas include alternative means of guidance, multiplexing devices so that more experiments can be done in parallel, incorporating electrodes within devices, and enabling other modes of injury within devices. There has been evidence that non-uniform AC fields can be used to dynamically control axonal outgrowth *in vitro* within microfluidic devices [195]. This phenomenon can be investigated within our tissue-level platform to examine if this is phenomenon is repeatable in an environment that more closely represents the *in vivo*. Incorporation of advances in high-throughput patterning can be used to create massively parallel arrays of single axons [196]. Patterned 3D neural networks are another exciting area of study, and extension of this technology to tissue level systems can provide an additional way to mimic the in *vivo* three-dimensional environment [197]. Another exciting evolution of both the cellular and organotypic devices can include the incorporation of microelectrode arrays which would enable functional studies of regenerating neurons by allowing for stimulation and recording of neuronal activity. The feasibility of these types of microfluidic arrays at both the two- and three-dimensional level within microfluidic neuronal culture and tissue culture has been recently demonstrated, and the natural progression to incorporate these within an injury platform would allow for studies that confirm if regenerating axons within these devices are quantifiably functional [198, 199]. Development of the organotypic

nerve injury device to incorporate other modes of injury would also be valuable. For example, in a recent brain-on-a-chip device, stretch injuries were modeled by applying culturing brain slices on a flexible substrate and applying a pressure underneath [200]. This technique can be extended to our two-compartment platform, which contains a flexible PDMS base that is easily modifiable.

Continuing to advance three-dimensional *in vitro* models of nerve injury and regeneration would enable development of future clinical therapies for improving quality of life and regain of function for those suffering from peripheral nerve injuries. Next generation devices are expected to continue to improve *in vivo* representation, as basic science promotes the development of advanced devices and advanced devices promote the development of cellular discoveries. Related advances in three-dimensional tissue and organ printing, for example, have additional exciting implications for a bottom up approach to developing accurate *in vitro* models. Future developments in the area of peripheral nerve injury research are expected to allow for a deeper understanding of the mechanisms that underlie both degeneration and regeneration, and enable the development of clinical therapies for enhancing functional recovery throughout the nervous system.

Appendix A

Appendix

A.1 Photolithography

Step	Process	Duration
Degrease and Dehydration Bake	Do a degrease (Acetone, IPA, N2 Dry) followed by 200°C dehydration bake for 10 minutes	~12-13 mins
Cool	Bring down to RT	~10 min
Plasma Treat	200 W, 3 mins exposure for each wafer	~10-15 mins
SU-8 2002	Center wafer, Program (step 1: 500 RPM, Accel: 85, time: 6s step 2: 900 RPM, Accel: 255,time:50s), Apply SU-8 to coat entire wafer, Run Program	~10-15 mins
Soft bake	Hot Plate: RT ramp to 65ºC (1 minute), then ramp to 95ºC (14 min including ramp)	15 mins
Cool	Ramp down to RT slowly	20 min
Exposure	Load bottom mask in aligner, Parameters = Hard Contact, Substrate: 0.50 mm, Resist: 3 µm, Separation: 50 µm, Dose:350 mJ/cm2	~30 min
Post-Expose Bake	Hot Plate: RT ramp to 65ºC (1 minute), then ramp to 95ºC (14 min including ramp), ramp down to RT	~25-30 mins
Develop	Immerse in developer (1-2 min), rinse with IPA, spin dry (2 min), inspect, repeat if necessary	~5 mins
Bake	45°C for 5 mins	5 mins
Plasma Treat	Descum with 300 mTorr, 75 W 20 seconds	20 s
SU-8 3050	Center wafer, Program (1500 RPM, Accel: slow, time: 60s), Pour half-dollar amount of SU-8 onto wafer, Run program	~5-15 mins
Softbake	Hot Plate: RT ramp to 65ºC (1 minute), then ramp to 95ºC (75 min including ramp)	~80 mins
Cool	Bring down to RT slowly and cool	~30 mins
Exposure	Load top mask in aligner, use alignment marks, Parameters = Soft Contact, Substrate: 0.50 mm, Resist: 90 µm, Separation: 250 µm, Dose: 450mJ cm2	~30 mins-120mins
Post-Exposure Bake	Hot Plate: RT ramp to 65ºC (1 minute), then ramp to 95ºC (14 min including ramp)	15 mins
Cool	Ramp down to RT slowly	20 mins
Develop	Immerse in developer (~5 min), sonicate if necessary, rinse with IPA, Spin dry (2 min), inspect	~15 mins
Hard Bake	Hot Plate: RT-->65ºC (1) -->95ºC (4) -->150ºC (overnight)	~12-15 hours
Cool	Bring down to RT slowly and cool	~30 min

A.2 Soft Lithography

1. Silane treat master wafer under vacuum (for microfluidic devices)
2. Mix Sylgard 184 (Polydimethylsiloxane (PDMS))in a ratio of 10:1 base:cross-linker for at least 5 minutes, vigorously until well aerated.
   (a) For microfluidic chambers: 50g base: 5g cross-linker
   (b) For single and dual chamber organotypic devices 20g base: 2g cross-linker
3. De-gas the aerated mixture in desiccator for 15-20 minutes
4. Open desiccator to remove remaining bubbles. Repeat degassing if necessary.
5. Pour mixture onto wafer or mold
6. Cover and let sit for 5 minutes to remove any bubbles from pouring.
7. Place in oven and bake at 85°C
   (a) For microfluidic chambers: 1-2 hours
   (b) For organotypic devices: 1 hour
8. Cool and remove PDMS from wafer/mold. For organotypic devices, place PDMS back into oven to continue baking to remove impurities for at least 72 hours
9. Cut out devices, clean, and plasma bond

A.3 Plasma Treatment

1. Ensure devices are clean
   (a) If microfluidic chambers, tape clean
   (b) Place in sonicator in 100% ethanol, channel side up (the side that will bond to glass up)
   (c) Set to sonic, time: 5 minutes
   (d) Remove from ethanol near hood, place on foil to dry
2. If microfluidic chambers, tape clean again if necessary or to ensure tight bonding
3. Open vent to plasma machine
4. Place device channel side up (or flat surface up for organotypic devices) glass slide or glass bottom dish (sterile and sonicated in ethanol)inside plasma machine
5. Close door to plasma chamber, close vent, turn on vacuum pump and pressure gauge, and ensure that oxygen tank is open
6. Let pressure come down to below 150 mTorr
7. Slowly open gas valve to increase pressure to around 500 mTorr
8. Turn on plasma, and switch power to high

9. Check that plasma light is purple in color (reflects energy level of plasma). Adjust pressure if necessary

10. Set timer to 1 minute and 25 seconds

11. Turn off plasma when timer runs out, turn off pump, turn off gas, and vent the chamber taking care not to open valve too fast

12. Work quickly once exposed to atmosphere, flip device over and attach onto exposed side of glass substrate, press to bond

13. For organotypic devices, plasma treat bonded devices once more for sterility bake for 5 minutes immediately post bonding to ensure strength of bond. For microfluidic devices, can bake 20 minutes to ensure strong bonding, or utilize temporary hydrophilicity immediately post bonding to coat devices with ECM

A.4 Spinal cord and Peripheral Nerve Co-Culture

1. Obtain mouse pups (p3 to p5 age range)
2. Prepare 70% ethanol solution for submerging dissection tools
3. Decapitate first pup with scalpel
4. Obtain nervous tissue sample from decapitated head and place on microscope slide
5. Check tissue under fluorescent scope to confirm fluorescence if using YFP mice
6. Pin down pup supine
7. Obtain median an ulnar nerves from each arm of pup before proceeding to spinal cord dissection
   (a) Remove superficial skin on arm to locate nerves
   (b) Dissect median and ulnar nerves from brachial plexus to wrist (as far as possible)
   (c) Place nerves in dissection media, separate, and clean
8. Excise volar skin

9. Cut through ribs bilaterally approaching and remove organs in order to expose spinal cord
10. Pin back any tissue or organs at tail to increase visibility
11. Carefully open spinal canal, taking care not to damage inner spinal cord tissue
12. Once bone is removed, run forceps carefully down each side of cord to avulse roots
13. Carefully life spinal cord tissue from caudal end and continue to excise roots as lifting up and moving distally
14. Place cord in dissection medium
15. Carefully dissect off dura
16. Place spinal cord on plastic transparency for use on tissue chopper
17. Ensure that tissue chopper blade is installed correctly and is parallel to the chopper plate and perpendicular to the spinal cord
18. Ensure spinal cord is relatively straight
19. Check that the tissue chopper is set to 350 micron thick slices
20. Adjust chopper start point so that entire cord will be cut in one run
21. Start machine and hold plastic down during run

22. Once cord is cut, invert plastic sheet into dissection media, rolling spinal cord sections into contact with media to ensure that they come off

23. Separate any sections that do not naturally separate and ensure that sections sink instead of float as floating will dry out sections

24. Cut a p200 pipette tip so that opening is large enough for entrance of a spinal cord section

25. Set pipette to approximately 150 μL to pull in individual sections and place them directly on a membrane

26. If required, move spinal cord into place

27. Identify ventral horn by locating central canal (ventral horn is closer to central canal)

28. Place a nerve at each ventral horn, abutting the cut nerve end to the spinal cord as closely as possible

29. Within two days, can check health of motor neurons

30. Allow enough time for adequate reinnervation of nerve (approximately 1 week)

31. Perform nerve injury by performing a transection in culture

32. Perform nerve repair by placing new wild type nerve next to transected nerve ends

A.5 Coating Devices for DRG cultures

1. Dilute either Poly-L-Lysine (PLL) or Poly-D-Lysine (PDL) to 1 mg/mL stock solution in ultra pure water

2. Freeze unused PLL or PDL in 1 mL aliquots

3. Dilute solution further to 100 μg/mL to obtain working concentration

4. Clear channels of the device

   (a) Apply ethanol to one side of device, let flow through channel and enter other compartment of device. Do a minimum of 3 washes with water (alternating sides) to ensure removal of ethanol

   (b) Alternatively, due to potential toxicity of residual ethanol, utilize hydrophilicity immediately post bonding to encourage coating of device for next step

5. Apply either PLL or PDL to one side of device, keeping one side of device slightly elevated to encourage flow

6. Let solution flow through channels and coat for at least 4 hours at room temperature or overnight at 4°C

7. Remove solution and replace with laminin in PBS (or ultra pure water) at a concentration of 10 μg/mL (good range between 1-10 μg/mL)

8. Let coat at least 4 hours in incubator

9. Remove laminin, wash 3x (alternating sides) with PBS, and do final wash with media

10. Prior to cell loading, remove media from from both sides, leaving enough to continuously coat the surface of the device, and load cells

A.6 Thin Collagen Gel Sheet Fabrication

1. Use a PDMS mold with a well roughly 50-100 microns deep depending on thickness needs
2. Sterilize PDMS mold with ethanol, 20 minutes
3. Perform all solution preparation on ice under sterile conditions
4. Mix Collagen I, Rat tail (Gibco), 7.5% sodium bicarbonate and 10X PBS in a 8:1:1 ratio
5. If laminin required, add 10 μg/mL to solution
6. Vortex solution
7. Place solution on ice for 20 minutes before loading solution into PDMS molds
8. Load gel with p200 pipet, starting in 200 μL increments
9. Place gels in incubator at 37°C for 2 hours to ensure adequate gelation
10. Additional time may be necessary to dry gels. Note that once mold is partially removed, gels will dry very quickly and may become difficult to handle
11. Once gels are stiff enough to handle, cut gels to necessary dimensions from formed sheets and place under transected nerve

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